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14. ABSTRACT

DAB2IP was identified to be a DOC-2/DAB2 interactive protein from normal basal cell population of prostate. DAB2IP is a new member of RAS-GAP family that is known to modulate extracellular signal-elicited RAS pathways. In androgen-independent prostate cancer (AIPCa), RAS activation is often detected while DAB2IP is down regulated due to epigenetic control such as DNA methylation and histone acetylation or methylation. Genetic variation of DAB2IP is associated with the risk of aggressive PCa. In DAB2IP-knock out mouse, prostate hyperplasia is also noticed. Biochemical studies demonstrate that DAB2IP has a similar function as PTEN to modulate cell survival and apoptosis by interacting with critical proteins involved in these pathways. Thus, DAB2IP is a homeostatic complex controlling cell growth, differentiation and survival of prostate epithelia.

15. SUBJECT TERMS
Cell differentiation, GTPase activating protein, tumor suppressor, Epigenetic regulation, signal transduction, prostate cancer

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INTRODUCTION

It is known that androgen-independent (AI) prostate cancer (PCa), a life threatening disease expresses many proteins associated with stem cells (1-4). In normal prostate gland, basal cell is considered as the stem cell because it maintains the homeostasis of prostate epithelial growth and differentiation into luminal epithelial cells (5-8). In AIPCa, loss of homeostatic control in these cancer cells contributes to the malignant phenotype.

To characterize homeostatic machinery in prostate stem cell, we identified a novel protein-DAB2IP that is a new member of RAS-GTPase activating protein (GAP) family. An increased c-H-Ras mRNA expression in PCa is associated with the progression of PCa to androgen independence (9). In addition to the GAP domain, DAB2IP (10) contains several other functional motifs such as a pleckstrin homology domain (aa 20-70) with a high affinity to certain phosphoinositides, a C2 domain (aa 90-120) involved in binding phospholipids in a calcium-dependent or -independent manner, a proline-rich (PR) domain (aa 796-805) involved in interacting with proteins that contain a SH3 domain, and a leucine zipper (aa 842-861), which mediates dimerization. In the past years, we have unveiled an altered epigenetic mechanism underlying the loss of DAB2IP in AIPCa, cloned mouse DAB2IP gene and generated knock out mouse with hyperplasia phenotype, and delineated the modulating role of DAB2IP in signaling cascades leading cell growth/survival/apoptosis. The outcome of this project has provided the role of DAB2IP in cell differentiation/growth of prostatic epithelium versus AIPCa. This knowledge should not only provide new understanding of stem cell also help to invent new therapeutic regimes for this disease.

BODY

Task 1. To delineate mechanism(s) leading to the down regulation of hDAB2IP gene in PCa and correlate the status of the hDAB2IP in PCa specimens with disease progression.
In this project, we unveiled the mechanism action of Ezh2, a histone methyltransferase often elevated in metastatic PCa (11-12), in suppressing hDAB2IP gene expression. This is the first report to detail the underlying mechanism of Ezh2 in PCa. Also, we also unveiled that Ezh2 can recruit HDAC to form a transcription repression complex. These data clearly indicate that the role of epigenetic control of tumor suppressor gene during PCa progression. In addition, we also report the hypermethylated DAB2IP promoter is detected in not only AIPCa cells and specimens but also other cancer cell types such as breast cancer as well.

A common genetic mutation of tumor suppressor genes is found in PCa, our data clearly indicate that epigenetic alteration is an alternative mechanism involved in gene silencing. Unlike genetic changes, epigenetic modifications are potentially reversible, which can open a new avenue of cancer therapy. Recently, we have reported that a Histone deacetylase inhibitor FK228 can be a potent therapeutic agent in treating AIPCa (13).

Task 2. To study the function role of hDAB2IP protein complex in prostatic epithelial differentiation.
Prostate homeostasis relies on a balance between cell proliferation, apoptosis and differentiation. Androgens, and more specifically dihydrotestosterone (DHT), are essential but are not directly responsible for prostatic growth as it has been supported by observations such as cultured normal

epithelial cells isolated from basal cell do not respond to androgen and prostate stromal cells with androgen receptor (AR) under androgen stimulation produce andromedin (14) that is a potent mitogen for prostate epithelia. On the other hand, androgen also appears to be a morphogen (15-17) because androgen is required for architect, secretory function and survival of luminal cell, indicating that androgen can orchestrate the effects of growth stimulatory and differentiation factors via paracrine and/or autocrine manner. Indeed, cross talks between different signaling axes are increasingly regarded as fundamentally important to the development, growth and maintenance of homeostasis in the prostate. In contrast, androgen becomes a potent mitogen for PCa, indicating the normal physiologic function of androgen is impaired in the malignant cells. Based on our study, DAB2IP appears to be a part of homeostatic machinery to modulate normal growth and differentiation of prostatic epithelium. Our preliminary data from DAB2IP knock out (KO) animal, we noticed that there is epithelial hyperplasia detected in 12-month old KO mouse compared to wild type mouse (Fig. 1). These data provide further support for the role of DAB2IP in prostate epithelial homeostasis.

Fig. 1 Prostatic hyperplasia is associated with DAB2IP-KO mouse. **A,** schematic representation of the configuration of DAB2IP KO gene and its PCR primer set. **B and C,** Southern analysis of DAB2IP gene. **D,** western analysis of wild type and KO mouse. **E,** immunostaining of DAB2IP expression in wild type and KO mouse. **F,** H&E staining of 12-month old wild type and KO mouse.
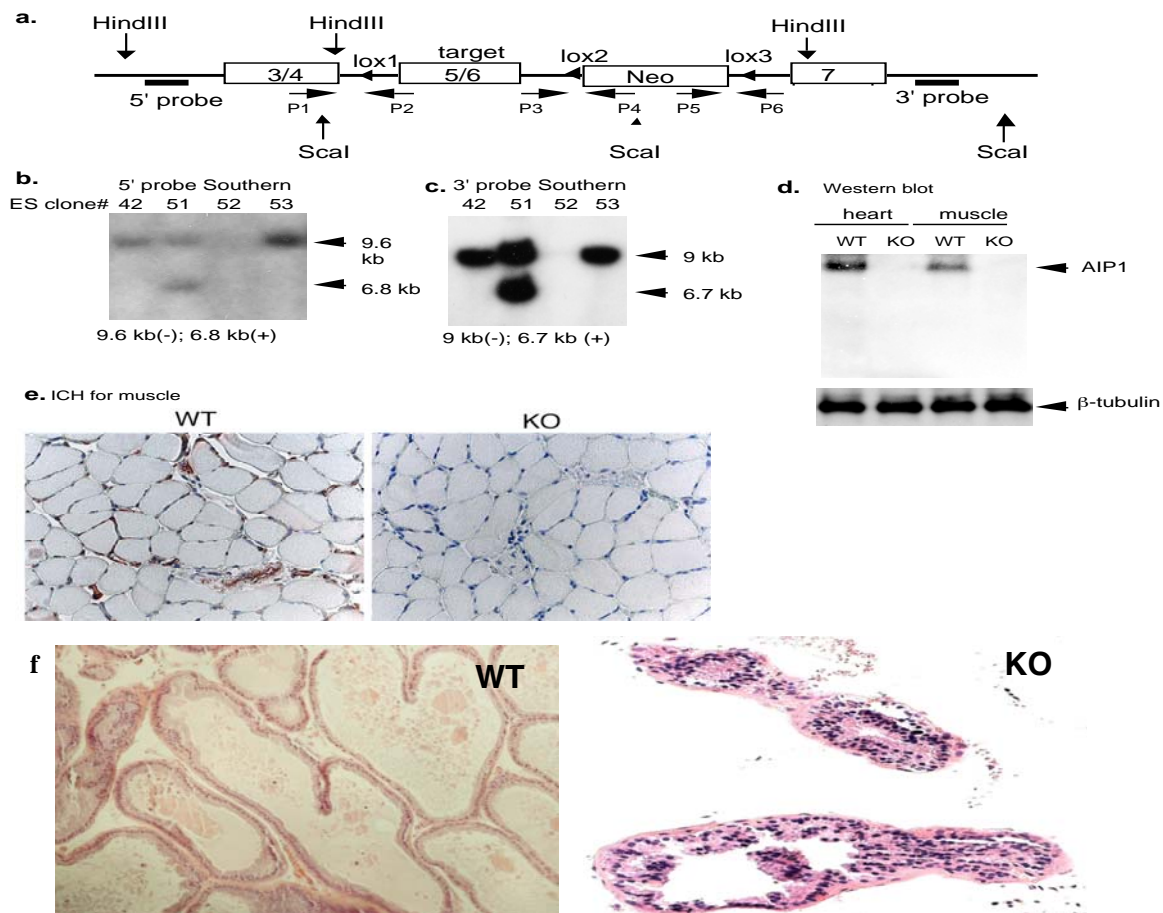
*Task 3.* To delineate signal cascade mediated by hDAB2IP protein complex in prostate epithelium.

The role of DAB2IP in modulating cell survival and death

Cell homeostasis is a balance between cell proliferation, apoptosis and differentiation in basal and luminal epithelia. It is believed that loss of homeostatic control in these cells renders the onset of neoplasm in prostatic epithelium. Until now, a little is known about homeostatic machinery in normal prostatic epithelium. Recently, we identified DAB2IP as a new member of GTPase activating protein (GAP) family as a potent growth inhibitor in metastatic PCa cell lines (10) by inducing G_0 cell cycle arrest and promoting apoptosis under stress factor (Fig. 2). It is known that the C2 domain can interact with ASK1 to facilitate TNF-α-mediated apoptosis by activating ASK1. Our preliminary data further indicated that the PR domain in DAB2IP is critical for binding PI3K regulatory subunit (p85) and then suppresses the activation of Akt (Fig. 3); the first six amino acids of PR domain appear to be key binding site (Fig. 4). It appears that DAB2IP-mediated Akt inactivation can concomitantly enhance ASK1 activation leading to cell apoptosis. In contrast, DAB2IP siRNA can restore Akt activity, suppress ASK1 activation and reduce apoptosis (Fig. 5), indicating that DAB2IP is critical factor for interaction between Akt and ASK1. Taken together, we believe that DAB2IP is a novel scaffold protein for both survival and death signal molecules, by which DAB2IP complex is a key machinery to maintain homeostasis in normal cell.

Figure 2 The role of DAB2IP in PI3K inhibitor (LY294002)-induced cell apoptosis. DAB2IP-transfected PCa lines (C4-2) were determined for DAB2IP expression (a), then the total cell number (b), the percentage of apoptotic cell (c) and apoptotic hallmark-PARP cleavage (d) were determined after 24 hrs of treatment.
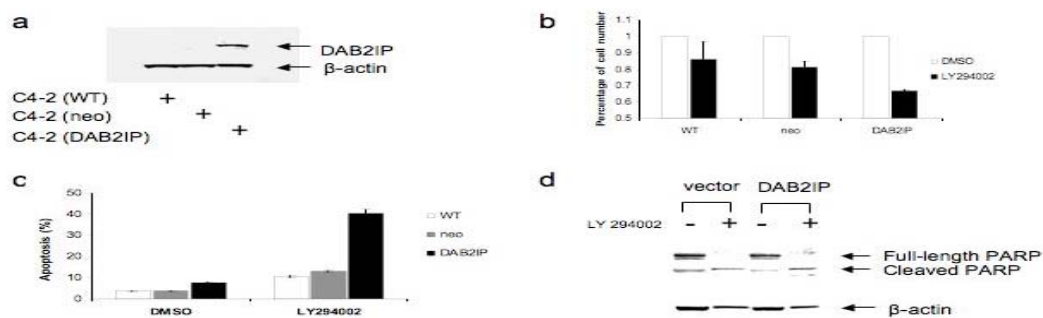

Figure 3 The level of phosphorylated Akt and total Akt in DAB2IP-transfected C4-2. Cells were treated LY294002 (10 μM) (a) or TNF-α (100 ng/ml) (b) for 30 min and total cell lysate
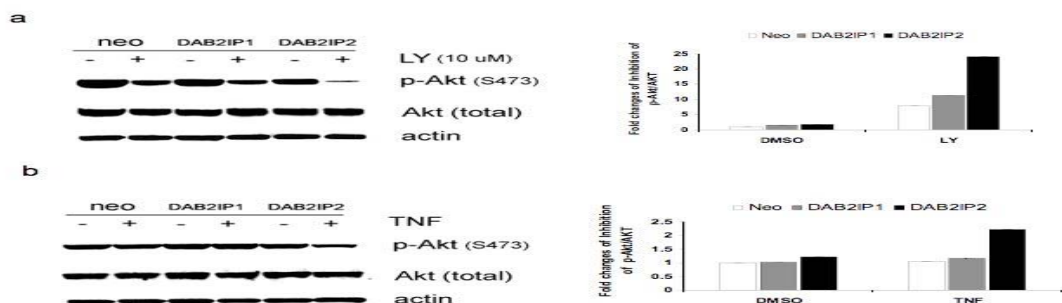
Figure 4 Determination of interactive domain in DAB2IP with the regulatory domain PI3K (p85). Using pull down (a) or immunoprecipitation (b), the first six amino acids in PR domain of DAB2IP is a critical binding domain with p85. The mutant can diminish the activity of DAB2IP in LY294002- or TNF-α-elicited cell apoptosis.
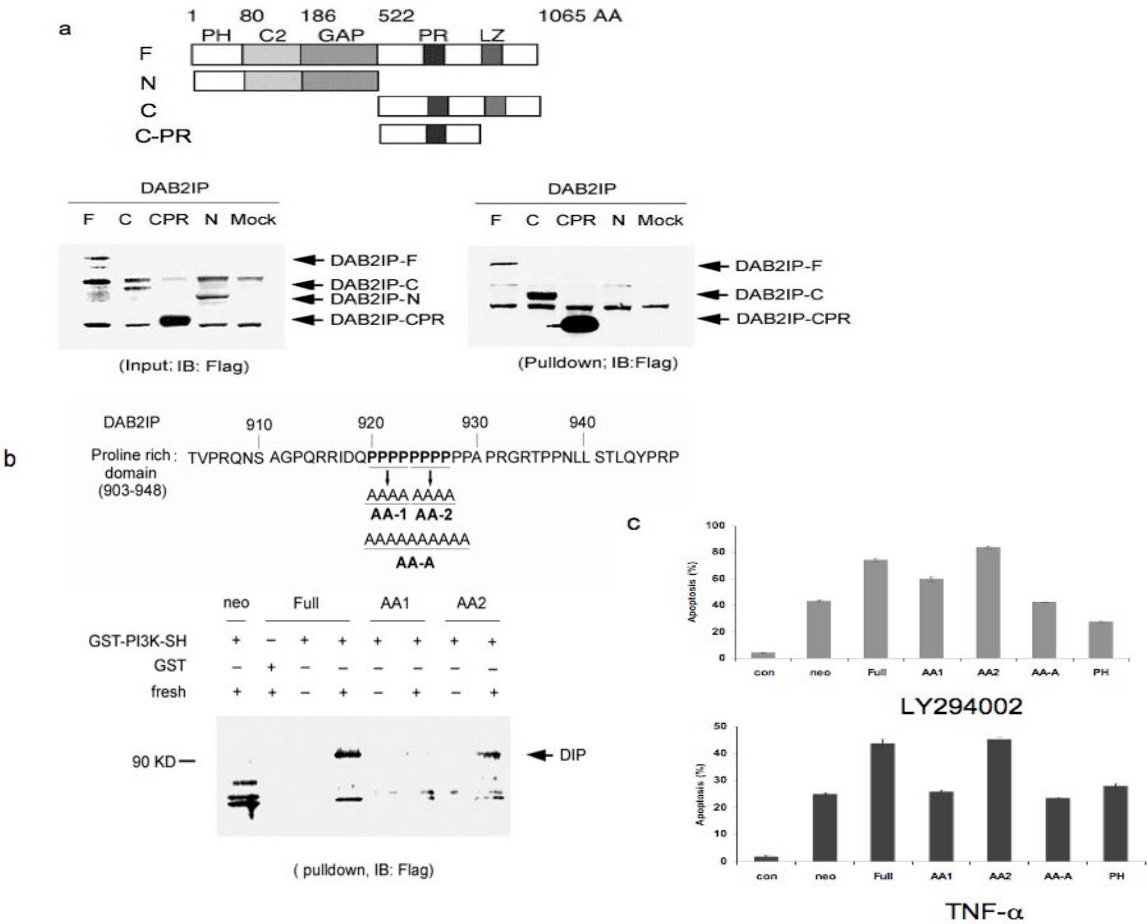
Figure 5 Reduction of LY294002- or TNF-α̃-mediated cell apoptosis by DAB2IP siRNA. Endogenous DAB2IP levels in PZ-HPV7 was decreased by DAB2IPsiRNA (a), which resulted in elevated activated Akt (b), decreased activated ASK1 (c) and decreased cell apoptosis (d̃) after treating with LY294002- or TNF-α.
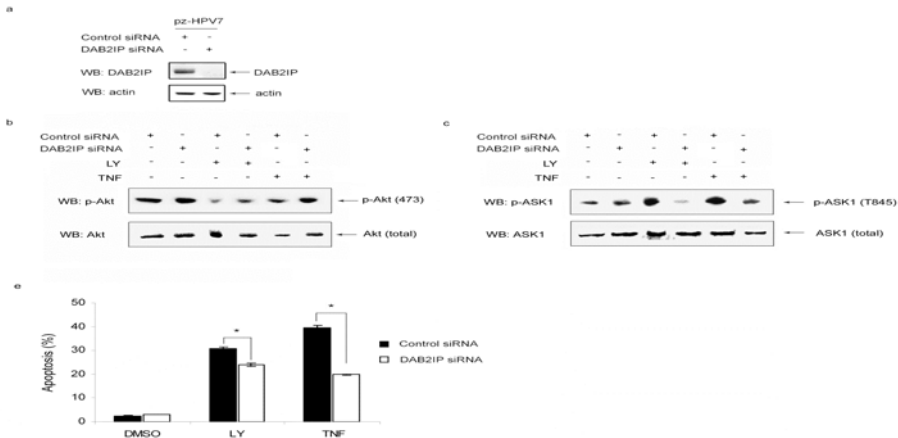
KEY RESEARCH ACCOMPLISHMENT

• Characterize the role of histone methylation in modulating hDAB2IP gene expression in PCa cells.

• Characterize the elevated Ezh2 levels correlated with down regulation of hDAB2IP gene expression in PCa.

• Delineate the role of Ezh2 in suppressing hDAB2IP gene expression in normal prostatic epithelia.

• Characterize the DNA methylation status of hDAB2IP gene promoter in PCa cell lines and specimens.

• Cloning and characterization of mouse DAB2IP gene and its promoter.
(**This work has been published on the cover page of DNA and Cell Biology journal**)

• Profile the DAB2IP gene expression in various mouse organs and PCa cells.

• Determine the epigenetic regulation of mouse DAB2IP gene in mouse PCa cells.

• Generate a DAB2IP KO mouse.

• Determine the possible role of DAB2IP in TNF-α-elicited cell apoptosis of PCa cells.

• Demonstrate the interaction between ASK1 and DAB2IP in PCa cells induced by TNF-α.

• Generate a series of DAB2IP mutant constructs.

• Map a new functional domain in DAB2IP for controlling cell survival.

• Determine the role of DAB2IP in cell survival/ apoptosis of PCa cells by various exogenous stimuli.

REPORTABLE OUTCOMES

FULL-LENGTH PAPER

1. Dote, H., Toyooka, S., Tsukuda, K, Yano, M., Ouchida, M., Doihara, H., Chen, H., Hsieh, J.T., Gazdar, A.F., Shimizu, N. (2004) Aberrant promoter methylation in human DAB2 interactive protein (hDAB2IP) gene in breast cancer. Clin. Cancer Res., 10, 2082-2089.

2. Chen, H, and Hsieh, J.T. (2005) Down regulation of human DAB2IP gene expression mediated by polycomb Ezh2 complex and histone deacetylase in prostate cancer. J. Biol. Chem., 280: 22437-22444.

3.   Zhou, J., Tu, S., Hernandez, G., Scholes, J., Chen, H., Tseng, C.P., and Hsieh, J.T. (2005) Synergistic induction of DOC-2/DAB2 gene expression in transitional carcinoma cells in the presence of GATA6 and histone deacetylase inhibitor. Cancer Res., 65: 6089-6096.

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REVIEW PAPER

1. Karam J., Benaim, E., Chen, H., Pong, R.C., and Hsieh, J.T. (2005) Epigenetics in prostate cancer. In Prostate Cancer: Mechanisms, Prevention, and Hormone Therapy (Chang, C., ed) World Scientific, pp 213-228.

2. Hsieh, J.T., Karam, J.A., and Min, W. (2007) The Link of Genetic and Biologic Evidence from DAB2IP gene with Aggressive Prostate Cancer. J. Natl. Cancer Inst., 99:1823-1824.

CONCLUSIONS

Basal cells of the prostate gland, considered as a stem cell population, are responsible for maintaining homeostasis of the normal prostate. Some evidences suggest AIPCa may arise from a prostate stem cell. The progression to AIPCa during androgen ablation therapy has led to speculation that PCa may contain a small population of AI cells that survive and can expand in the absence of androgen. It is known that basal/stem cell in prostate is androgen independent, normal prostate stem cell and AIPCa may share the common properties. For example, Collins *et al* (18) demonstrated that the primary human PCa with the highest *in vitro* proliferative potential is CD44+$\alpha_2\beta$1hiCD133+ and androgen receptor (AR)-negative subpopulation, which is noticed in normal prostate basal cell population (19). Thus, we believe that loss of homeostatic control in AIPCa contributes the onset of this disease and identification of this machinery will lead to new strategy to target this disease.

DAB2IP was identified to be a DOC-2/DAB2 interactive protein associate with normal basal cell population of prostate. DAB2IP is a new member of RAS-GAP family that is known to modulate extracellular signal-elicited RAS pathways. In AIPCa, RAS activation is often detected while DAB2IP is down regulated due to epigenetic control such as DNA methylation and histone acetylation or methylation. A recent study from Genomic Wide Association (20) indicated the presence of single nucleotide polymorphism in DAB2IP gene that could predispose patients to aggressive PCa. In DAB2IP-KO mouse, prostate hyperplasia is also noticed. In addition, DAB2IP has a similar function as PTEN to modulate cell survival and apoptosis by interacting with critical proteins involved in these pathways. Thus, DAB2IP is a homeostatic complex controlling cell growth, differentiation, and survival of prostate epithelia.

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APPENDIX

1. Xie, D., Zhang, H., Yu, L., Min, W., and Hsieh, J.T. (2008) DAB2IP, a novel tumor suppressor, modulates PI3K/Akt and ASK1 pathways (In preparation).

Appendix 1

DAB2IP, a novel tumor suppressor, modulates PI3K/Akt and ASK1 pathways

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Running Title: DAB2IP modulates cell survival and apoptosis

Summary

Constitutive activation of phosphatidylinositol 3-kinase (PI3K)-Akt/PKB and inactivation of apoptosis-stimulated kinase (ASK1)-JNK pathways are often detected in metastatic prostate cancer (PCa) cells. Here, we identified DAB2IP is, often down-regulated in PCa, a potent growth inhibitor by inducing G_0 cell cycle arrest and promoting apoptosis in metastatic PCa cell lines. Biochemical studies demonstrate that DAB2IP has a similar function as PTEN because the proline-rich (PR) domain can interact with PI3K and inhibit its activity also the PER domain particularly S604 is critical for suppressing Akt activation. DAB2IP-mediated PI3K-Akt inactivation can simultaneously enhance ASK1 activation leading to cell apoptosis. In contrast, DAB2IP siRNA can restore Akt activity and suppress ASK1 activation, which results in reducing apoptosis in normal cell under stress. Therefore, DAB2IP is a novel scaffold protein for both survival and death signal molecules, by which DAB2IP complex is a key machinery to maintain homeostasis in normal cell.

Significance

Imbalance of apoptotic and/or survival signaling cascade is a hallmark of malignant cell. In prostate cancer (PCa), constitutive activation of phosphatidylinositol 3-kinase (PI3K)-Akt/PKB and inactivation of apoptosis-stimulated kinase (ASK1)-JNK pathway signaling are often detected in metastatic cells. Previously, we have identified DAB2IP as a new member of GTPase activating protein (GAP) family. In this study, we further unveil that the DAB2IP is a novel scaffold protein to balance cell survival and apoptosis by modulating for PI3K-Akt/PKB and ASK1 activities. These data implicate DAB2IP as a potential therapeutic agent in PCa.

Introduction

Homeostatic balance between cell proliferation, survival and apoptosis is essential for ontogenesis. Any imbalance in these processes often leads to pathologic changes in cells, particularly, cancer malignancy. Many cancer cells in response to stimuli exhibit unscheduled and accelerated cell proliferation, unusual cell survival then escape apoptosis ultimately. Elucidating the underlying mechanism leading to such alternations will provide a better treatment strategy to control the terminal stage of this disease.

PI3-Kinase (PI3K)/Akt signaling pathway plays a critical role in cell proliferation and survival growth, and motility—processes that are critical for tumorigenesis. Indeed, the role of this pathway in oncogenesis has been extensively investigated and altered expression or mutations of many components of this pathway have been implicated in human cancer (Vivanco and Sawyers, 2002). PI3Ks is a heterodimer consisting of a 110-kDa catalytic subunit (p110) and an 85-kDa regulatory subunit (p85). The p85 regulatory subunit stabilizes the p110 catalytic subunit and maintains it in a low activity state (Yu et al., 1998). Activation of PI3K occurs via the binding of p85 to tyrosine-phosphorylated proteins, which serves both to relieve inhibition on the p110 catalytic subunit as well as to recruit PI3-kinase from the cytosol to the plasma membrane, where its substrate, PI-4,5-P2, resides (Rordorf-Nikolic et al., 1995). Akt is activated by phosphorylation at T308 and S473 following PI3K activation (Alessi et al., 1997). The mechanisms of overactivated PI3K/Akt signaling include protein overexpression (Moore et al., 1998; Ma et al., 2000; Cheng et al., 1992), mutations of Ras oncogenes (Okudela et al., 2004; Bloethner et al., 2005), loss of the tumor suppressor gene PTEN (Choe et al., 2003, Chang et al., 2003), which promote tumor cell proliferation and survival in a variety of cancers. It is known that Akt has

many cellular targets including ASK1 (Kim et al., 2001). ASK1, a member of the MAPK kinase kinase (MAP3K) family, is an upstream activator of JNK and p38 MAPK signaling cascades (Davis 2000). Although the activation of ASK1 triggers apoptosis in response to diverse stress and apoptotic stimuli such as TNF-a or reactive oxygen species (Ichijo et al., 1997; Chang et al., 1998), ASK1 may also induce cell survival depending on the cell type and cellular context (Takeda et al., 2000). However, the underlying mechanism is not fully understood.

We previously identified DAB2IP/AIP1 (DOC-2/DAB2 interactive protein or ASK interacting protein 1) (Wang et al., 2002; Zhang et al., 2003), a new member of RAS-GTPase activating protein (RAS-GAP) family, as a potent tumor suppressor protein from a yeast two-hybrid system. Loss of DAB2IP expression mainly due to epigenetic regulation of its promoter is often found in androgen-independent PCa (Cheng et al., 2003; Cheng et al., 2005) as well as other cancer types (Dote et al., 2004; Dote et al., 2005; Yano et al., 2005; Qiu et al., 2007). In a recent study, single nucleotide polymorphism in DAB2IP gene could predict the risk of aggressive prostate cancer (PCa) (Duggan et al., 2007). DAB2IP consists of several conserved structural domains–the pleckstrin homology (PH), the protein kinase C conserved region 2 (C2) and GAP at the N-terminal half, and at the C-terminal half a period-like (PER) domain, a proline-rich (PR) domain and a leucine-zipper motif (LZ). DAB2IP can stimulate H-RAS, R-RAS and TC21 but not RAP1A GTPase activities and suppresses epidermal growth factor-elicited PCa growth (Wang et al., 2002). In addition to its GAP activity, DAB2IP is also involved in TNF-α-mediated cell apoptosis by facilitating dissociation of ASK1 from its inhibitor 14-3-3 via its PH and C2 domains (Zhang et al 2004; Zhang et al 2007). Obviously, DAB2IP is involved in different biologic functions in both malignant cell and normal cell.

Here, we unveil that DAB2IP causes cell cycle arrest and potentiates intrinsic or extrinsic apoptotic pathways. Structural functional analyses indicate that the PR domain is critical for modulating PI3K activity and the PER domain is critical for Akt activity. DAB2IP can coordinate PI3K/Akt inactivation with ASK1 activation, which results in diminishing cell survival and enhancing cell apoptosis in cancer cell. In contrast, knocking down endogenous DAB2IP in normal cell can attenuate cell apoptosis elicited by stress signal and PI3K inhibitor. Therefore, DAB2IP is a novel scaffold protein to interact with both survival and death signal molecules, by which DAB2IP complex is a key machinery to maintain homeostasis in normal cells.

Results

DAB2IP induces G_0/G_1 cell cycle arrest and promotes apoptosis

Our previous data showed that DAB2IP functions as a potent growth inhibitor for PCa cells *in vitro* (Wang et al., 2002). However, in addition to its GAP activity, the mechanism of action of DAB2IP is largely unknown. Thus, we examined whether DAB2IP might affect cell cycle progression. Using a DAB2IP-expressing C4-2 subline (i.e., D2), a tumorigenic PCa line without endogenous DAB2IP expression, and a vector-control subline (i.e, neo) (Wang et al., 2002), we compared cell cycle distribution between these sublines. As shown in Figure 1A, the percentage of G_0/G_1 cells ($79.1 \pm 1.4\%$) in D2 subline is about 20% more than that in neo subline ($58.9 \pm 2.7\%$). In contrast, the percentage of S or G_2/M phase in D2-subline is less than that in neo-subline, indicating that DAB2IP can elicit G_0/G_1 cell cycle arrest.

Although Cyclin D is maximally expressed following mitogenic stimulation of G_0 cells, Cyclin D remains high and invariable thorough the whole cell cycle in many tumor-transformed cell lines (Matsushime et al., 1994). Cyclin E shows a periodic pattern of expression with accumulation in

late G1 and downregulation in S phase, by which it activates CDK2 to drive the cell through S phase (Koff et al., 1991). We showed "unscheduled" pattern of cyclin D1 and cyclin E expression in C4-2-neo cells. Both cyclin D1 and cyclin E are not limited in G1 and S, but expressed throughout all the cell cycle phases including G2 and M phases, which is consistent with observation in several other solid tumor cells (Gong et al., 1994; Gong et al., 1995). To further validate the effect of DAB2IP on G_0/G_1 arrest, bivariate analysis of cellular DNA with cyclins expression such as cyclin D1 and E using flow cytometry (Darzynkiewicz et al., 1996) was carried out. In D2 subline, both cyclin D1 and E protein levels decreased in either G_0 or early G_1 phase (Figure 1A); this result is consistent with cell cycle analysis that the expression of DAB2IP can decrease G_1 cyclins levels underlying G_0 or early G_1 cell cycle arrest.

In Figure 1A, a slightly increased sub-G_1 was noticed in D2 subline. Also, previous data indicate that DAB2IP can facilitate TNF-α-elicited apoptosis mediated via apoptosis signal-regulating kinase 1 (ASK1) activation in endothelial cells (Zhang et al., 2003). Thus, DAB2IP could be pro-apoptotic for PCa cells as well. Apoptosis is known to be mediated through two major signaling pathways — the 'intrinsic' (mitochondrial) and the 'extrinsic' (death receptor) pathways (Ashkenazi et al., 2002; Ghobrial et al., 2005). To determine the effect of DAB2IP on apoptosis, PI3K inhibitor LY294002 (LY) and TNF-α were used to represent the mitochondrial and death receptor pathways respectively (Kulik et al., 2001). In this experiment, both transient and stable DAB2IP-transfected C4-2 cells were used. In transient transfected cells treated with LY, there are less viable cells in DAB2IP-expressing (0.66 ± 0.01) than VC (vector control) cells (0.81 ± 0.03) (p<0.01, **Figure S1**). Western blot analysis of PARP cleavage was much evidenced in DAB2IP-transfected cells (Figure 1B). Annexin V/PI staining data indicated that the number of

apoptotic cell increased from 7.6±0.87% to 33.6±2.1% in DAB2IP-transfected cells under LY treatment, which is almost three times higher than VC cells (from 5.56±0.85% to 14.5±3.2%) (p<0.01, Figure 1B). Based on these results, DAB2IP is able to enhance LY-elicited apoptosis. In addition to LY, we also examined TNF-α effect on two stable DAB2IP-transfected cells (i.e., D1, D2). The percentage of apoptotic cells induced by either LY (D1: 30.2±0.82%; D2: 40.3±0.98%) or TNF-α (D1: 45.8±0.73%; D2: 58.2±1.74%) is significantly higher than neo cells (13.06±0.68% for LY; 21.3±1.39% for TNF-α) (p<0.01, Figure 1C), which is correlated with the levels of DAP2IP protein expression (Figure 1C). Also, DAB2IP-enhanced cell death is dependent on caspase pathways because the presence of pan-caspase inhibitor (Z-VAD) can abolish DAB2IP effect (Figure 1C).

To determine if endogenous DAB2IP regulates apoptosis, knocking down endogenous DAB2IP in an immortalized normal prostate epithelium (PZ-HPV7) cells was performed. Loss of DAB2IP protected cell apoptosis which decreased approximately 3 folds in LY- and 2 folds in TNF-α-treated cells compared to control siRNA (i.e., Lamin A/C) cells (Figure 1D). Collectively, these findings confirm that DAB2IP is a potent cell cycle inhibitor and pro-apoptotic factor to potentiate intrinsic or extrinsic apoptotic pathways.

DAB2IP inhibits PI3K/Akt and actives ASK1 activity

To unveil the mechanism of action of DAB2IP in these events, the activation status of Akt (S473) or ASK1 (T845) was determined based on the specific phosphorylation site of each protein. Comparing with neo cells (Figure 2A, lane 2), there is a dramatic inhibition of Akt phosphorylatin in D1 and D2 cells under LY treatment (p<0.01, Figure 2A, lane 4 and 6). On the other hand, ASK1 activation is significantly elevated in both D1 and D2 cells than that in neo cells

(approximately 3 folds, p<0.01, Figure 2B), indicating that DAB2IP can activate ASK1. Similar findings were observed in D1 and D2 cells under TNF-α treatment (Figure 2C and D). In contrast, under the same treatment condition, an increased Akt activation and ASK1 inhibition were detected in PZ-HPV7 cell when its steady-state levels of DAB2IP were knocked down by siRNA (Figure 2E). Thus, DAB2IP appears to be a novel modulator to bridge both PI3K/Akt and ASK-mediated pathways.

DAB2IP binds to the regulatory domain of PI3K (p85) through PR domain

Based on the predicted functional domain in DAB2IP protein, the PR domain in DABIP could bind to the Src homology 3 (SH3) domain found in the regulatory subunit of PI3K (p85). To test the possibility of such interaction, both *in vitro* pull-down and *in vivo* co-IP assay were carried out. Using GST-p85-SH3 fusion protein as a bait, various DAB2IP expression vectors including Full length (F; amino acid 1-1065), N-terminal (N; amino acid 1-522), C-terminal (C; amino acid 523-1065), and C-PR (CPR; amino acid 523-805) were transfected into 293 or C4-2 cell (Figure 3A and 3B). From *in vitro* pull-down assay, it appears that DAB2IP-F, DAB2IP-C and DAB2IP-C-PR, but not DAB2IP-N can bind to GST-p85-SH3, suggesting that the PR domain is able to bind to p85-SH3 domain.

From *in vivo* co-IP assay, although there is a weak interaction between DAB2IP and p85 or ASK1, dramatic increased DAB2IP and p85 complex is detected in 293 cells under LY treatment (Figure 3C). In this complex, the active form of ASK1 (T845) is more visible than the inactive form of ASK1 (S83) (Figure 3D). Concurrently, a decreased active form of Akt (S473) is found in this complex following LY treatment (Figure 3D), suggesting that DAB2IP can enhance the effect of LY on Akt inhibition via a direct protein direction. **Similar pattern was also detected in 293 cells**

under TNF-α treatment (data not shown). Clearly, DAB2IP is a unique scaffold protein that binds to p85 through PR domain and ASK1 through C2 domain respectively to modulate cell survival or death signal pathways.

Structurally, DAB2IP contains unusual ten repeats of proline in the PR domain. To further map the binding site, three different DAB2IP mutant constructs (AA1: first four-prolines to alanines; AA2: second four-prolines to alanines; AAA: all ten-prolines to alanines, Figure 4A) were examined. From a pull-down assay, DAB2IP-AAA completely abolishes the binding to p85 (Figure 4B). However, DAB2IP-AA1 appears to have more impact on p85 binding than DAB2IP-AA2, indicating that the first four proline residues is the critical binding site for p85. Similarly, using co-IP, DAB2IP-AAA mutant fails to interact with p85 in 293 cells under either LY (Figure 4C) or TNF-α (Figure 4D) treatment.

PR domain in DAB2IP dictates PI3K/Akt inhibition and ASK1 activation

To examine whether the formation of DAB2IP and p85 complex has any effect on catalytic subunit (i.e., p110) of PI3K, immunocomplex of p110 was prepared from 293 cells transfected with DAB2IP-F or DAB2IP-AAA after LY or TNF-α treatment then subjected to a non-radioactive competitive ELISA assay (Figure 5A). In this experiment, low concentration (10 μg/ml) of LY without significant PI3K inhibition compared to the positive control of LY at 80 μg/ml was used.   As shown in Figure 5A, DAB2IP-F could potentiate the inhibitory effect of LY or TNF-α on PI3K activity, however, DAB2IP-AAA mutant lost such activity. This result is parallel with both the p85 binding (Figure 4C and 4D) and the status of Akt inactivation (Figure 5B). To elucidate the mechanism of DAB2IP effect on PI3K inhibition, tyrosine phosphorylation on p85 was determined by phos-antibody, and interaction between p85 and p110 was determined

by immunoprecipation. DAB2IP-F has no effect on effect on p85 tyrosine phosphoryaltion (Fig S2). However, the association of PI3K catalytic domain (p110) with p85 and DAB2IP-F is elevated under LY or TNF-α treatment and DAB2IP-AAA mutant blocks such interaction (Figure 5C). It is known that Akt can phosphorylate ASK1 leading to ASK1 inactivation (Kim et al., 2001). As expected, in the DAB2IP-F-expressing cells, the active form of ASK1 (T845) increases significantly compared with the vector control. However, the levels of ASK1 activation significantly reduce in DAB2IP-AAA mutant cells (Figure 5D) due to its inability of inactivating Akt (Figure 5B).

ASK1-mediated S604 phosphorylation in DAB2IP is critical for binding to Akt

We have previous showed that phosphorylation of S604 in DAB2IP is critical for its activation (Zhang et al., 2003). To determine whether S604 is important for the binding of DAB2IP with Akt, cell lysates from 293 cells transfected with DAB2IP-F and DAB2IP-S604A mutant following LY or TNF-α treatment were subjected to western blot with phospho-DAB2IP-S604 antibody or immunoprecipitated with Akt antibody. As shown in Figure 6A and 6B, both treatments clearly increase S604 phosphorylation in DAB2IP and this site of phosphorylation is critical for the interaction of DAB2IP with Akt.

To define the candidate kinase for S604 phosphoryaltion, ASK1 was examined since the increased association of ASK1 with DAB2IP under LY or TNF-α treatment. By co-transfecting DAB2IP-F or DAB2IP-S604A mutant with either wild type (WT) or kinase **defective** (KR) of ASK1 in 293T cells, DAB2IP-S604 phosphorylation was detected by DAB2IP-phospho-antibody (S604). ASK1-WT can increase S604 phosphoralytion in DAB2IP, however, ASK1-KR fails to phosphorylate S604. In DAB2IP-S604A-expressing cells, a substantial decreased phosphorylation

at S604 is observed (Figure 6C). These data suggest that S604 in DAB2IP is a substrate for ASK1 and this site is critical for DAB2IP activation.

Akt binds to the PER domain in DAB2IP

Considering that S604 is within the PER domain in DAB2IP and the function of this domain is not well characterized, this domain was screened for any binding sites of potential interactive proteins based on Scansite program (Figure S3), and Akt at S604 turned to be one of candidates. To confirm this result, two deletion mutants of PER domain (C-PER [aa 522-719], C-tPER [aa 522-620], Figure 7A) were generated and subjected to co-IP assay. From the total cell lysate, expression levels of DAB2IP-F or mutants are similar, noticeably, S604 phosphorylation is only detected in cells transfected with DAB2IP-F, DAB2IP-CPER and DAB2IP-C-tPER but not in DAB2IP-S604A vectors (Figure 7B). From the result of co-IP (Figure 7C), Akt binding site appears to be within aa 620-719 in DAB2IP. In addition, DAB2IP-S604A mutant fails to associate with Akt, suggesting that S604 phosphorylation may contribute for the unfolding of DAB2IP (Figure S4). To further identify whether the binding of Akt to DAB2IP could alter Akt activation, western blot analysis using phospho-Akt (S473) antibody was carried out. It appears that the degree of inhibitory effect of Akt (S473) phosphorylation from DAB2IP is DAB2IP-F > DAB2IP-CPER ≈ DAB2IP-S604A > DAB2IP-C-tPER in 293 cells under LY or TNF-α treatment (Figure 7D).

PR and PER domain are critical for DAB2IP-mediated apoptosis

To correlate the structural functional relationship of DAB2IP in PCa, C4-2 cells were transfected with various DAB2IP mutants following LY or TNF-α treatment. The data indicated that DAB2IP-AAA or DAB2IP-S604A mutant could diminish DAB2IP-enhanced apoptosis while

DAB2IP-AA1 mutant exhibited less effect and DAB2IP-AA2 mutant had no effect (Figure 8A and B). In addition, DAB2IP-R289L, a GAP mutant (Wang et al., 2002), does not impair DAB2IP effect on enhancing apoptosis (Figure 8A and 8B), indicating that GAP domain is not involved in this event. Taken together, in response to various exogenous or endogenous stress signals, the change of DAB2IP conformation could function as a novel scaffold protein to modulate key effector molecules involved in either cell survival or apoptosis in normal or cancer cells (Figure 8C).

DISCUSSION

Altered homeostatic control in cell proliferation, survival and apoptosis underlies many diseases particularly cancer malignancy. In many cancer types including PCa (Lin et al., 1999), PI3K/Akt signaling axis has been described as a dominant survival pathway (Vivanco et al., 2002). In contrast, insufficient activation of ASK1, an important mediator of apoptotic signaling responses to many stress stimuli, and/or its downstream MKK4-JNK-c-Jun pathway has been correlated with aggressive tumor development of PCa (Kim et al., 2001). It appears that there is a cross talk between PI3K/Akt and ASK1 pathways in which Akt can phosphorylate ASK1 resulting in inactivation of ASK1 (Okubo et al., 1998; Levresse et al., 2000; Galvan et al., 2003). However, it remains to be examined in the context of other ASK1 regulators (Kim et al., 2001).

In cancer cells, tumor suppressor genes normally restrict cell proliferation/survival or promoting apoptosis (Macleod, 2000 and Sherr, 2004). As shown in previous studies, DAB2IP can modulate cell proliferation (Wang et al., 2002) and also can bind then active ASK1 during TNF-α-mediated apoptosis (Zhang et al., 2003); these data support the tumor suppressive function of DAB2IP. In this study, we further demonstrate the additional function of DAB2IP to bridge PI3K/Akt and

ASK1 pathways to maintain balance between cell survival and death pathways. Functionally, DAB2IP is similar to PTEN to suppress PI3K/Akt pathway, however, DAB2IP is a scaffold protein rather than phospholipase that forms a complex with various effector molecules. Thus, re-expression of DAB2IP in PCa cell becomes pro-apoptotic since DAB2IP is able to enhance cell apoptosis in the presence of extrinsic or intrinsic stimulus (Figure 1B, 1C and S1). Consistently, knock down of endogenous DAB2IP in normal cells (Figure 1D) renders cells become apoptosis resistance.

The p85, a regulatory subunit of PI3K, contains SH3, BCR and two SH2 domains. Under resting conditions, p85 serves to stabilize p110 protein and inhibit PI3K kinase activity (Yu et al., 1998). This inhibitory effect is alleviated by binding of the SH2 domain of p85, particularly the NH2-terminal SH2 domain, to cell membrane tyrosine-phosphorylated receptors, tyrosine-phosphorylated peptides, or linker molecules containing the YXXM motif (Songyang et al., 1994). This inhibitory effect presumably due to the conformational changes in the p85–p110 complex might involve the SH3 and BCR domains (Vivanco and Sawyers, 2002). Thus, the SH3 and BCR domains are postulated to have a negative regulatory role towards the catalytic activity of p110 subunit (Ueki et al., 2000; Vivanco and Sawyers, 2002). Our results (Figure 3-5 and S2) indicate that the unique PR domain with ten-proline-repeat in DAB2IP binds to the SH3 domain of p85; the first eight-proline is critical for the binding. This interaction has no effect on p85 phosphorylation, however, it dictates inhibition of PI3K activity and subsequent Akt inactivation by recruiting both p110 and Akt respectively. We have demonstrated that DAB2IP relocates from membrane to cytosol in the presence of TNF-α (Zhang et al., 2004). Apparently, DAB2IP serves

as an anchorage for p85-p110 complex to dissociate from membrane activation site. These data

provide further evidence for the role of SH3 domain of p85 in inhibiting PI3K activity.

Data from this study conclude that DAB2IP functions as a key merging point for various

signals-elicited pathways. For example, TNF-α treatment results in Akt inactivation because

DAB2IP recruits PI3K and Akt protein via its PR and PER domain (Figure 2, Fig 7) respectively.

LY treatment results in ASK1 activation because DAB2IP recruits ASK1 protein via C2 domain

(Zhang et al., 2003). It is known that TNF-α-elicited ASK1 activation requires several sequential

steps including TRAF-dependent homodimerization/polymerization (Gotoh et al, 1998), ASK1

autophosphorylation at T845 (Tobiume et al., 2002) and release of inhibitors (thioredoxin and

14-3-3) (Saitoh et al., 1998; Zhang et al, 1999). For DAB2IP, it can dissociate inhibitor 14-3-3

from binding to the C-terminal of ASK1 via a lysine-rich cluster within the N-terminal C2 domain

(Zhang et al., 2003). In addition, the inhibitory effect of DAB2IP on PI3K/Akt is required for

ASK1 activation (Figure 5D) because AKT is able to inactivate ASK1 by phosphorylating S83 of

ASK1 or suppress the ASK1-JNK pathway by phosphorylating MLK3 (Barthwal et al., 2003) and

MKK4 (SEK1) (Park et al., 2002) from a variety of cell systems. Conversely, data from this study

(Figure 6, 7) indicate that ASK1 activation is able to inhibit PI3K/Akt-mediated pathways in

which DAB2IP also plays a central role because S604 phosphorylation in DAB2IP can be induced

by ASK1. Although S604 as a direct substrate for ASK1 needs further investigated, the S604

phosphorylation level once is induced by LY or TNF-α, the binding ability of DAB2IP to Akt or

p85-p100 complex increases as well (Figure 4-7, S4), suggesting the phosphorylation of S604 site

is very likely related with conformational changes of DAB2IP from inactive to active status. One

lead for supporting this prediction is the location of S604 in PER domain, which is a critical

binding site for Akt protein (Figure 7B, 7C). Also, we observed a similar induction of S604 phosphorylation in DAB2IP in other cancer cell than PCa, implying a general function of this protein in different cell types (Figure 2, 6, 7, S5). Taken together, we believe that DAB2IP forms a "signal circle" to counter balance between cell survival and apoptosis.

Very likely, each functional domain in DAB2IP has unique functions and yet acts coordinately. For example, the GAP activity of DAB2IP is known to be a negative regulator for Ras-Raf-ERK activation critical for cell growth (Wang et al., 2002). We noticed that re-expression of DAB2IP in PCa has significantly increased G_1 subpopulation (Figure 1A). From the bivariate analysis of cellular DNA content vs. expression of cyclins using multiparameter cyclins/DNA flow cytometry (Figure 1B), decreased both cyclin D1 and E were detected in DAB2IP-expressing PCa cells, which further confirm that DAB2IP can elicit G0/G1 cell cycle arrest. Recent studies showed EZH2-stimulated ADRB2 repression and downregulation of Rap1-GTPase activities involves induction of an EMT in prostate cancer cells (Yu et al., 2007). Interesting, DAB2IP is another GAP repressed by EZH2 (Chen et al., 2005). The effects of EZH2's repression of DAB2IP-GAP activity on the epithelial phenotype need to be determined. Thus, DAB2IP appears to be a unique factor in modulating both cell survival, growth and death by inhibiting the Ras-Raf-ERK pathway, PI3K/Akt pathway and activating ASK1-JNK/p38 apoptotic signaling pathways, by which DAB2IP complex is a key machinery to maintain homeostasis in normal cells. In conclusion, DAB2IP is a part of homeostatic machinery to modulate cell proliferation, survival and apoptosis in normal cell.

Experimental Procedures

Cell culture and Transfections

C4-2 cells (WT, Neo, D1, D2) were maintained in T medium supplemented with 5% fetal bovine serum (Wang et al, 2002). PZ-HPV-7, an immortalized normal cell derived from the peripheral zone of a benign prostate (Weijerman et al., 1994), was maintained PrEGM medium (Cambrex BioScience, Walkersville, Inc.). Human embryonic kidney 293 cells were maintained Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% FBS and penicillin/streptomycin.

Cells (5×10^5 cells/well) were plated in a six-well plate (Costar, Acton, MA) with 70% to 80% confluence before transfection. The transfection was carried out using Lipofectamin PLUS (Invitrogen) according to the instructions of the manufacturer with total amount of DNA of 1 µg/well for 24 hours.

Plasmid Constructs and Antibodies

Various expression plasmids for DAB2IP, anti-DAB2IP polyclonal antibody and anti-phospho-DAB2IP-specific Antibody (S604) were described previously (Wang et al, 2002; Zhang et al, 2003; Zhang et al, 2004). Anti-FLAG was from Sigma (St. Louis, MO). Anti-PI3K-p85, anti-PI3K-p110, anti-Akt, anti-actin and anti-ASK1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (S473), anti-phospho-ASK1 (T845 or S83), and anti-phospho-p85 were obtained from Cell Signaling Technologies (Beverly, MA).

RNA Interference for DAB2IP

Three pairs of siRNA oligonucleotides for human DAB2IP (sense strand, 5'-GGAGCGCAACAGUUACCUGTT-3' [siRIP1-A]; 5'-GGUGAAGGACUUCCUGACATT-3' (siRIP1-B); 5'- GGACUUGUUUUUUGUCACATT-3' [siRIP1-C]), and control siRNA for Lamin A/C (5'-CTGGACTTCCAGAAGAACA-3') were synthesized by Invitrogen. siRNA (100 nM) was

transfected into cells using LipofectAMINE 2000 (Invitrogen) according to manufacturer's protocols.

Immunoprecipitation, pull-down assay, and western blot analysis

For immunoprecipitation, transfected 293 cells after LY294002 (LY) or TNF-α treatment were washed twice with cold phosphate buffered saline and lysed in 1.5 ml of cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA) for 20 min on ice. The immunocomplex was subjected to immunoblotting as described previously (Zhang et al., 2003; Zhang et al., 2004).

For GST pull-down assay, transfected cells were treated with LY or TNF-α for 30 min and cell lysate was prepared by incubating with 0.5 ml of lysis buffer for 20 min on ice. After a low speed spin, 0.4 ml of supernatant was separately incubated with either 30 µl of GST-glutathione-Sepharose or GST-PI3K (p85)-SH-Sepharose (Zhou et al., 2003) overnight at 4 °C. The next day, the pellet was washed twice with lysis buffer and dissolved in the sample buffer (AU: what's in sample buffer) and then subjected to western blot analysis.

PI3K activity assay

A non-radioactive competitive ELISA-based assay (Echleon Biosciences, Salt Lake City, UT) was used to assess the PI3K activity. Briefly, PI3K complex was prepared from the same amount of cell lysates by immunoprecipitated with anti-p110 antibody overnight at 4°C and then were incubated with protein A-Sepharose beads for 1h at 4°C. Bead-bound enzymes were incubated with 4 µM of PI(4,5)P2 substrate in kinase reaction buffer (4 mM MgCl$_2$, 20 mM Tris, pH 7.4, 10 mM NaCl and 25 µM ATP) for 2h at room temperature. The mixtures were then incubated with

PI(3,4,5)P3 detector protein for 1h at room temperature in the dark then added to PI(3,4,5)P3-coated microplate for incubating 30 min at room temperature in the dark. After thorough washing, peroxidase-linked secondary detector and colorimetric detection reagent were added, which can detect PI(3,4,5)P3 detector protein binding to the plate by measuring $OD_{450\,nm}$. The colorimetric signal is inversely proportional to the amount of PI(3,4,5)P3 produced by PI3K. Relative PI3K activity was calculated using untreated cells (=1).

Apoptosis and cell cycle analysis

Cells were harvested and fixed in ice-cold 80% ethanol at -20°C for at least 24h. Cells pellet were resuspended in 1 ml of 0.1% sodium citrate containing 0.3% NP-40, 0.2 μg/ml RNase, and 50 μg/ml propidium iodide (PI), and incubated for 30 min on ice (Riccard et al., 2006). The profile of cells in the sub-G_1, G_0/G_1, S, and G_2/M phases of the cell cycle were analyzed on a FACSCaliber with Cellquest Software (Becton Dickinson, Franklin Lakes, NJ). For Annexine-V/PI assay (Bender System, Vienna, Austria), cells were harvested and rinsed with PBS containing Ca^{2+} and Mg^{2+}, then resuspended in 490 μl ice-cold annexin V–binding buffer to 10^5 to 10^6 cells/ml. Annexin V/FITC and PI reagents were added into cell suspensions on ice as specified by the manufacturer and incubated on ice in the dark for 10 minutes, then analyzed by flow cytometry.

For Cyclins/DNA multiparameter assay (Gong et al., 1995), cells were fixed in ice-cold 80% ethanol at -20°C for at least 24h. Cell pellet was washed in PBS, and permeabilized with 0.5% Triton-X100 in PBS for 5 min on ice. After centrifugation, cells were incubated with primary antibody to cyclin D1 and cyclin E (BD PharMingen, San Diego, CA) diluted in PBS containing 1% BSA at 4°C overnight. Cells were then rinsed and incubated with the secondary FITC-conjugated antibody (BD PharMingen, San Diego, CA) diluted in PBS containing 1% BSA

at room temperature for 30 min. After resuspending with PI solution (50 μg/ml PI), cells were

incubated at room temperature for 30 min. Fluorescent intensity was measured using the

FACSCaliber with Cellquest Software (Becton Dickinson).

Acknowledgements

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Figure legends

Figure 1. DAB2IP induces G_0/G_1 cell cycle arrest and promotes apoptosis

(A) DAB2IP induces G_0/G_1 cell cycle arrest. Cell cycle distribution of exponential growing neo and D2 cells was detected by PI staining using flow cytometry. Subpopulations of cells in G_0/G_1, S, and G2/M can be identified, as shown, based on cellular DNA content. Asterisks indicate statistically significant in D2 cells compared with neo cells ($p < 0.01$). For analysizing cyclin E and D1 expression using cyclins/DNA multiparameter flow cytometry, the trapezoidal "window" represents the level of fluorescence of the isotype IgG control, indicating the range within which cyclin-negative cells are located.

(B) DAB2IP promotes apoptosis in C4-2 cells transiently transfectd with DAB2IP or control vector (VC) after LY treatment. Apoptosis was determined by western blot using PARP antibody or Annexin V/PI flow cytometry. Asterisk indicates statistically significant in cells transfected with DAB2IP vs VC ($p < 0.01$).

(C) DAB2IP promotes apoptosis in D1, D2 or neo cells treated with LY or TNF-α. Apoptosis was determined by flow cytometry. Asterisks indicate statistically significant in DAB2IP-expressing cell vs neo cell ($p < 0.01$).

(D) Knockdown of DAB2IP protects apoptosis in PZ-HPV7 cell. Cells were transfected with siRNA after LY or TNF-α treatment. Apoptosis was determined by flow cytometry. Asterisks indicate statistically significant in cells transfected with DAB2IP-siRNA vs Control siRNA ($p < 0.01$). All the Data (mean ± standard error) were generated from three independent experiments.

Figure 2. DAB2IP inhibits PI3K/Akt and activates ASK1

(A, B, C, D) Cells lysates were prepared from neo, D1, D2 cells after LY (A, B) or TNF-α(C, D) treatment, and the status of Akt or ASK1 activation was determined by western blot with phospho-specific antibodies of Akt (S473), ASK1 (T845) respectively. The same membrane was stripped and re-probed with total Akt or ASK1 antibody, by which quantification of relative kinases activation was normalized. Data (mean ± standard error) were generated from two independent experiments. Asterisks indicate statistically significant in DAB2IP-expressing cell vs neo cell (Fig 2A, 2B, 2D * p <0.05; Fig 2C * p <0.5, ** p <0.05).

(E) PZ-HPV7 cell transfected with siRNA as indicated then treated with LY or TNF-α. Cell lysates were subjected to western blot probed with the same antibodies and quantification method as described above. Data (mean ± standard error) were generated from two independent experiments. Asterisks indicate statistically significant in cells transfected with DAB2IP-siRNA cell vs Control siRNA (p <0.05).

Figure 3. DAB2IP binds to p85 via PR domain

(A, B) DAB2IP binds to p85 *in vitro*. After transfecting with different DAB2IP cDNA constructs, 293 (A) or C4-2 (B) cells were treated with LY for 30 min then cell lysates were subjected to pull-down assay using GST-p85-SH3 fusion protein, resolved on SDS-PAGE and probed with Flag antibody. In these experiments, 5% of each input lysate was used as a loading control.

(C) DAB2IP interacts with p85 or ASK1 *in vivo*. With the same transfection and treatment condition, lysates of 293 cells were subjected to co-IP using Flag, p85, or ASK1 antibody.

(D) DAB2IP complexes with active form of ASK1 and inactive form of Akt. 293 cells were tranfected with DAB2IP-F or VC. Cell lysates were Co-IP with anti-Flag antibody then probed

with phospho-ASK1 (T845 or S83) or phospho-Akt (S473) antibody.

Figure 4. The First four-proline in PR domain is critical for DAB2IP binding to p85

(A) Sequence depict of PR domain mutants in DAB2IP. The cluster of four prolines in DAB2IP is underlined and replaced with alanine to generate AA1, AA2, and AAA.

(B) Determination of DAB2IP binding site for p85 *in vitro*. 293 cells were transfected with DAB2IP-F, AA1, AA2, or AAA mutant following LY treatment. Cell lysates were pulled down with GST-p85-SH3 fusion protein, resolved on SDS-PAGE, and probed with Flag antibody.

(C, D) Mutation of prolines abrogates DAB2IP binding to p85 *in vivo*. 293 cells were transfected with DAB2IP-F and AAA mutant following LY (C) or TNF-α (D) treatment. Cell lysates were IP with Flag antibody and probed with p85 antibody.

Figure 5. PR domain in DAB2IP dictates PI3K/Akt inhibition and ASK1 activation

In all experiments, 293 cells were transfected with DAB2IP-F, AAA mutant or VC following LY or TNF-α treatment.

(A) DAB2IP inhibits PI3K activity. Cell lysates were IP using p110 antibody and the PI3K activity of each immunocomplex was measured by an ELISA-based assay. Relative PI3K activity was normalized with VC cells without treatment. VC cells treated with 80 μM LY for 30 min was used as a positive control. All the data (mean ± standard error) were carried out in triplicates.

Asterisks indicate statistically significant differences in cells transfected with VC or AAA vs DAB2IP ($p < 0.01$).

(B) DAB2IP dictates Akt inhibition. Western blot analysis was first detected by phospho-Akt (S473) then reprobed with Akt antibody, and relative Akt activation was calculated after normalizing band intensity of phospho-Akt with total Akt. Data (mean ± standard error) were generated from three independent experiments. Asterisks indicate statistically significant differences in cells transfected with VC or AAA vs DAB2IP ($p < 0.01$).

(C) DAB2IP enhances the formation of PI3K complex. Cell lysates were immunoprecipitated by anti-p85 and then immunoblotted with anti-p110. In DAB2IP-F cells without treatment, immunoprecipitated p110 was considered as a basal level (1x) and fold induction was calculated. Asterisks indicate statistically significant differences in cells transfected with DAB2IP-F vs AAA ($p < 0.01$).

(D) DAB2IP dictates ASK1 activation. Western blot analysis was first detected by phospho-ASK1 (T845) then reprobed with ASK1 antibody, and relative ASK1 activation was calculated after normalizing band intensity of phospho-ASK1 with total ASK1. Data (mean ± standard error) were generated from three independent experiments. Asterisks indicate statistically significant differences in cells transfected with VC or AAA vs DAB2IP ($p < 0.01$).

Figure 6. ASK1-mediated S604 phosphorylation in DAB2IP is critical for binding to Akt

(A, B) S604 phosphorylation is critical for the interaction of DAB2IP with Akt. Cell lysates prepared from 293 cells transfected with DAB2IP-F and DAB2IP-S604A mutant following LY (A)

or TNF-α (B) treatment were subjected to both western blot probed with phospho-DAB2IP or Akt antibody and Co-IP with Akt antibody then probed with Flag antibody.

(C) ASK1 phosphorylates S604 in DAB2IP. 293 cells co-transfected with DAB2IP-F or DAB2IP-S604A mutant with either ASK1 wild type (WT) or kinase inactive (KR; Lys 709 was replaced by Arg), and cell lysates were detected with phospho-DAB2IP, DAB2IP, or ASK1 antibody.

Figure 7. Akt binds to the PER domain in DAB2IP

(A) Schematic depict of PER domain mutants in DAB2IP. DAB2IP S604A (Ser 604 was replaced by Ala), DAB2IP-C-PER (amino acids 522–719) and C-tPER (amino acids 522–620) are indicated.

(B) 293 cells were transfected with DAB2IP-F, S604A, C-PER and C-t PER constructs following LY or TNF-α treatment. Cell lysates were subjected to western blot probed with Flag or phospho-DAB2IP antibody.

(C) Cell lysates were subjected to Co-IP with Akt antibody and probed with Flag antibody.

(D) Cell lysates were subjected to western blot first detected with phosphor-Akt antibody then probed with Akt antibody.

Figure 8. PR and PER domain are critical for DAB2IP-mediated apoptosis

(A, B) C4-2 cells were transfected with various DAB2IP mutants following LY or TNF-α treatment and apoptosis was determined as described previously.

(C) A model for DAB2IP function as a scaffold protein to balance cell survival and apoptosis. In

response to various stress signals (e.g. LY or TNF-α), the binding of ASK1 to DAB2IP eliciting S604 phosphorylation precedes DAB2IP activation. Activated DAB2IP, probably due to the conformation change, binds to p85 via PR domain. The p85 further recruits p110 to inhibit PI3K/Akt-mediated cell survival pathway.

Supplement Data

Figure S1. DAB2IP promotes cell death

C4-2 cells transfectd with DAB2IP or VC following LY treatment, and viable cells were determined by crystal violet assay. Data (mean ± standard error) were generated from three independent experiments. Asterisk indicates statistically significant difference in cells transfected with DAB2IP vs VC (p <0.01).

Figure S2. DAB2IP does not alter p85 phosphorylation

293 cells were transient transfected with DAB2IP-F, AAA mutant, or VC following LY treatment. Cell lysates were subjected to western blot analysis probed with Flag, phospho-p85 or p85 antibody as indicated. Data (mean ± standard error) were generated from three independent experiments. Asterisk indicates no statistically significant differences in cells transfected with DAB2IP or AAA vs VC (p >0.5).

Figure S3. Akt is a candidate protein binding to the PER domain in DAB2IP

Amino acid sequence of DABIP-PER domain (aa 591-719) was scanned using Scansite program. Akt binding site at S604 in DAB2IP is highlighted in red.

Figure S4. S604 phosphorylation influences DAB2IP binding to p85

293 cells were transfected with DAB2IP-F, S604A mutant following LY or TNF-α treatment. Cell lysates were subjected to both western blot probed with phosphor-DAB2IP antibody or Flag antibody and Co-IP with P85 antibody and probed with Flag antibody. In DAB2IP-F cells without treatment, immunoprecipitated P85 was considered as a basal level (1x) and fold induction was calculated. Data (mean ± standard error) were generated from two independent experiments. Asterisks indicate statistically significant differences in cells transfected with DAB2IP-F vs AAA (p <0.01).

Figure S5. S604 phosphorylation of Endogenous DAB2IP

Cell lysates prepared from Hela and PZ-HPV7 cells following LY or TNF-α treatment were subjected to western blot probed with phospho-DAB2IP and DAB2IP antibody.

Fig 1. DAB2IP induces G1/G0 cell cycle arrest and promotes apoptosis
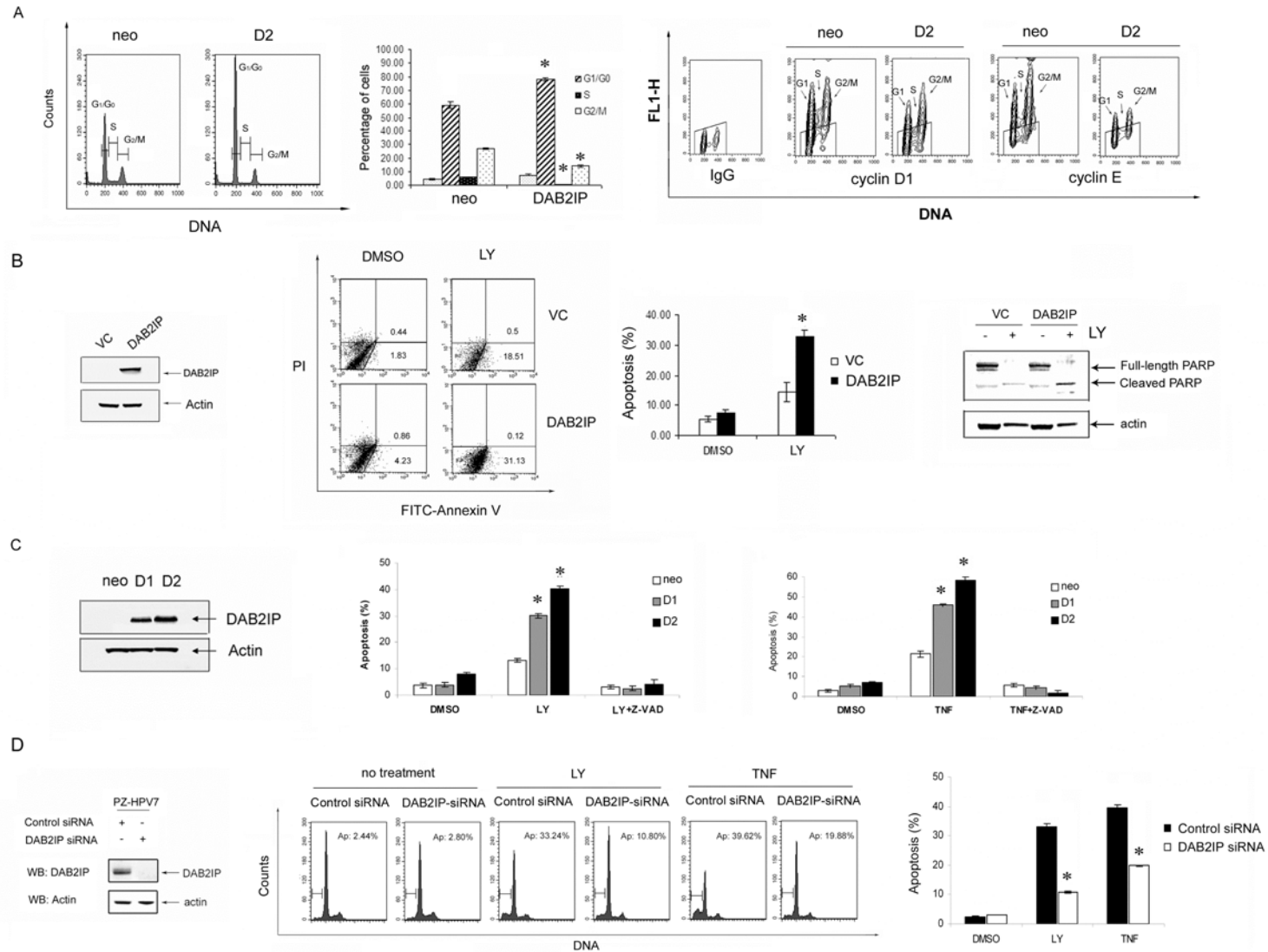
Fig 2. DAB2IP inhibits PI3K/Akt and active ASK1 activity
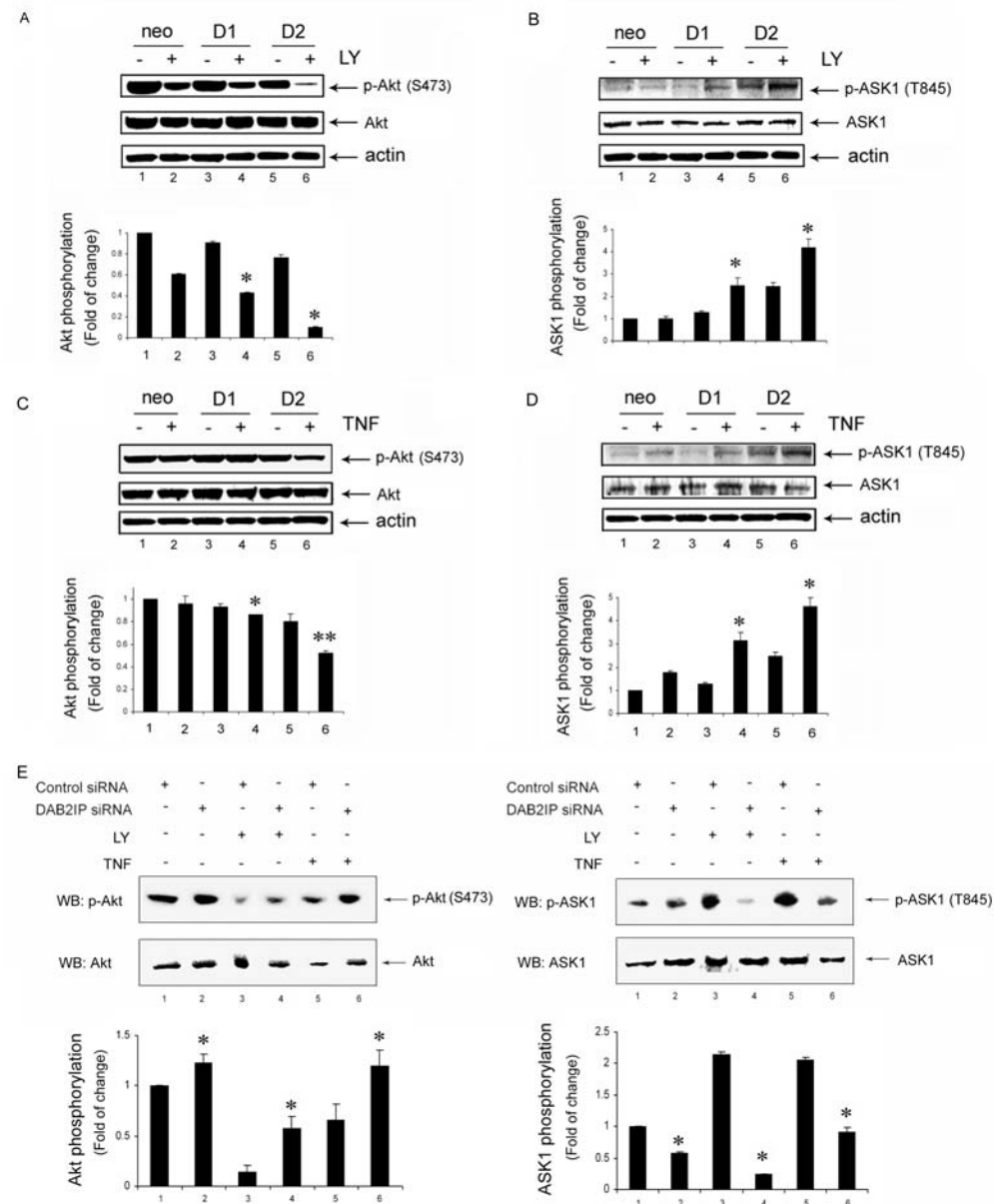
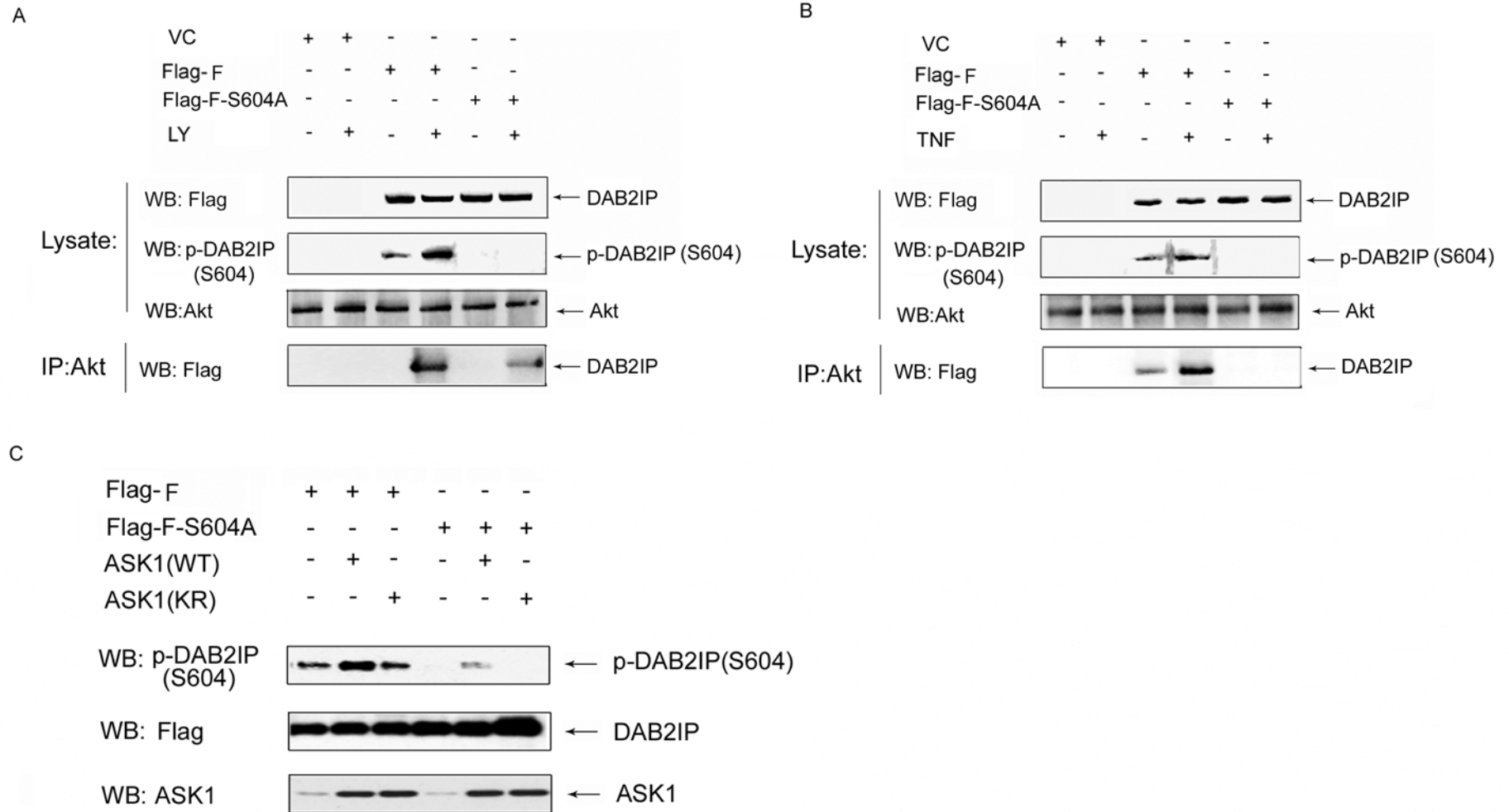# Fig 3. DAB2IP binds to the regulatory domain of PI3K (p85) through PR domain
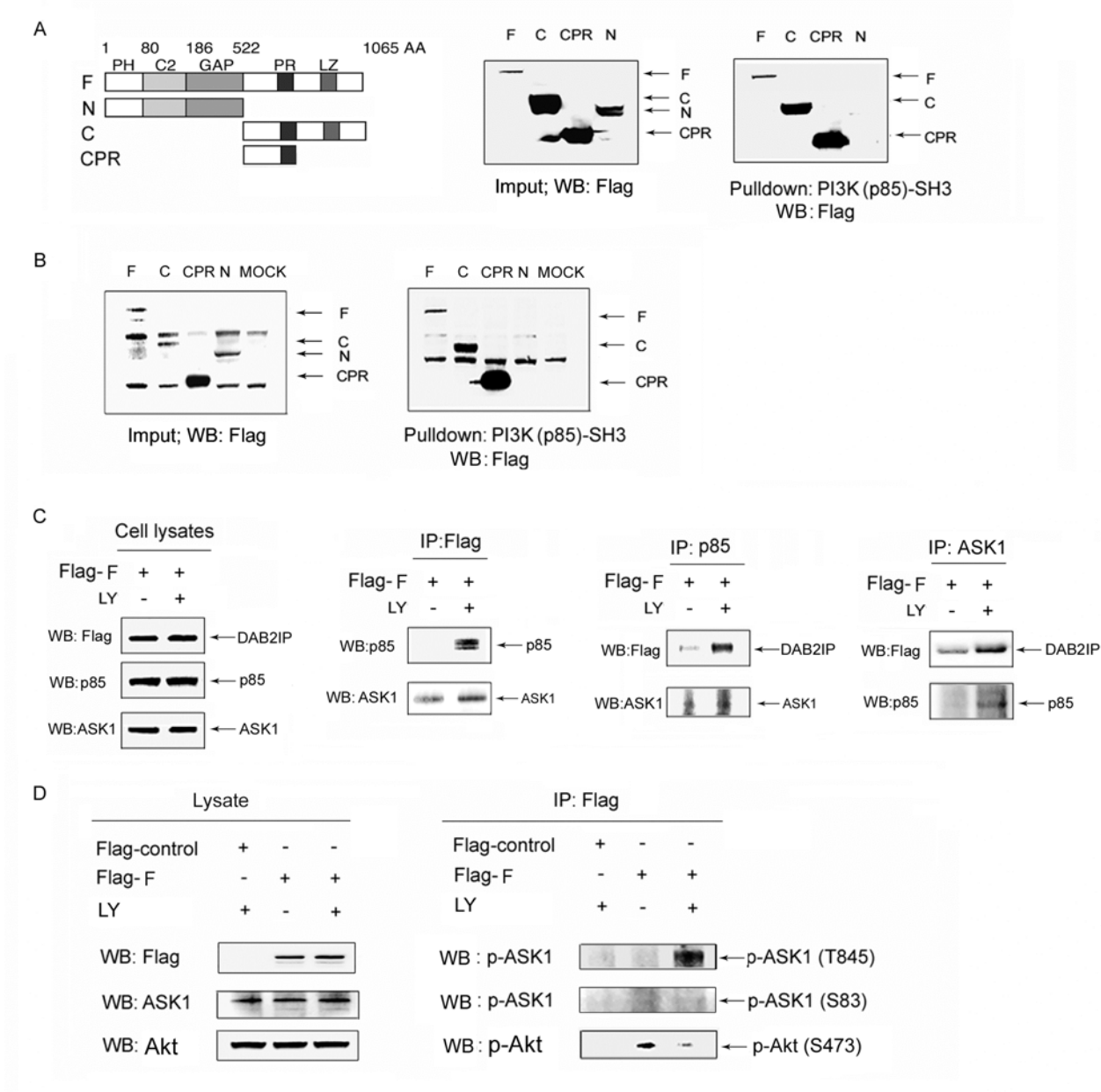
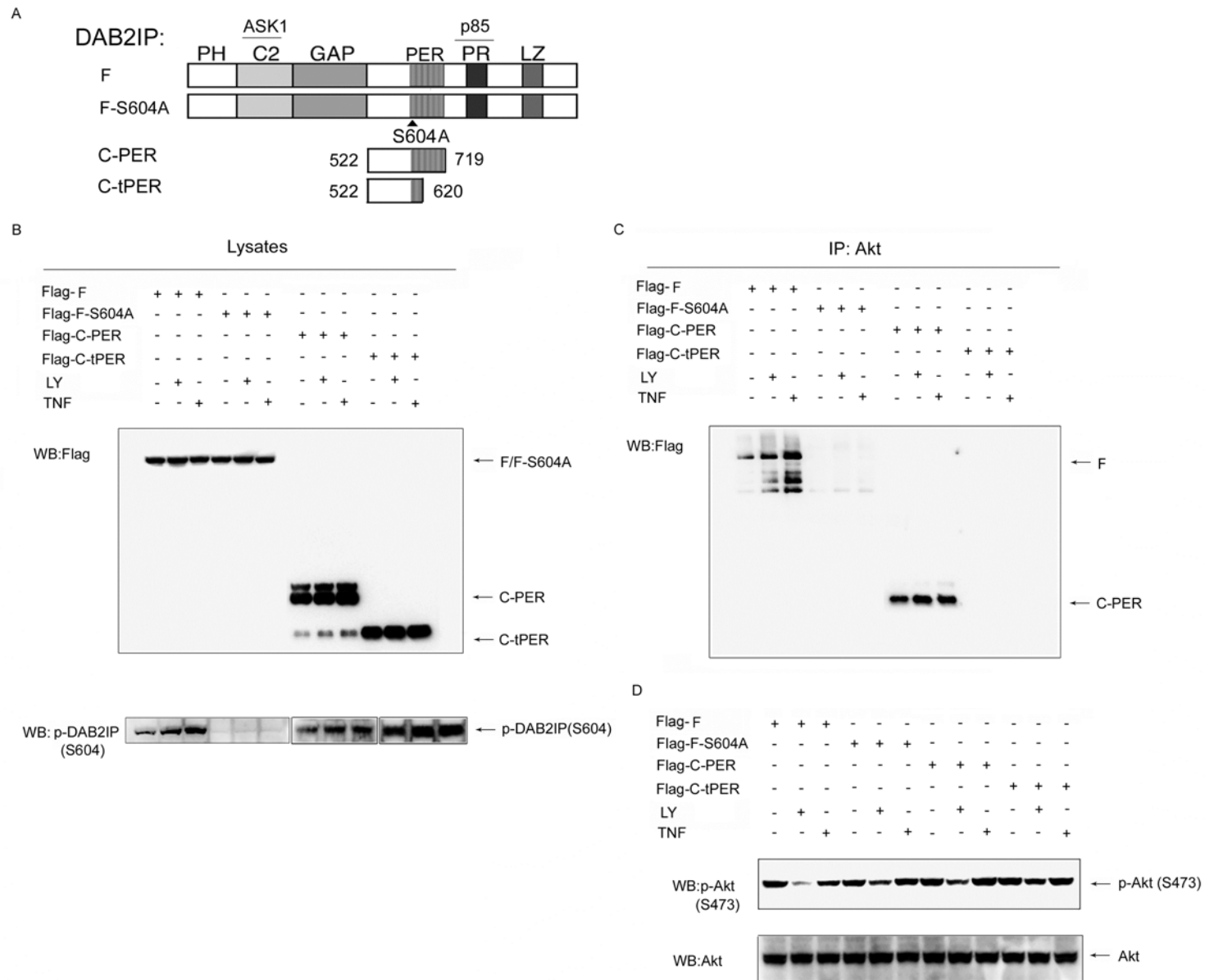# Fig 4. First four-prolines in PR domain is critical for DAB2IP binding to p85
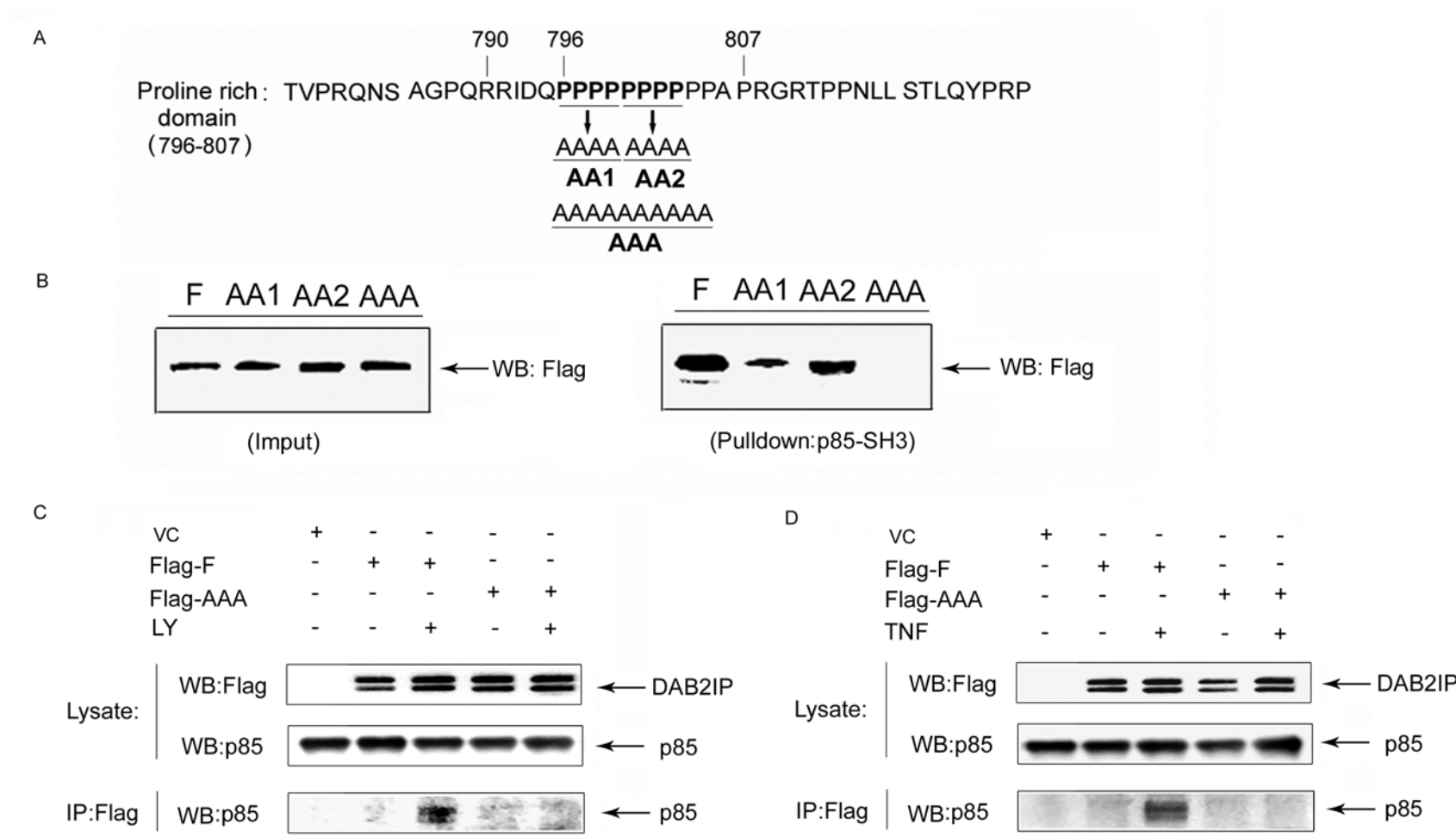
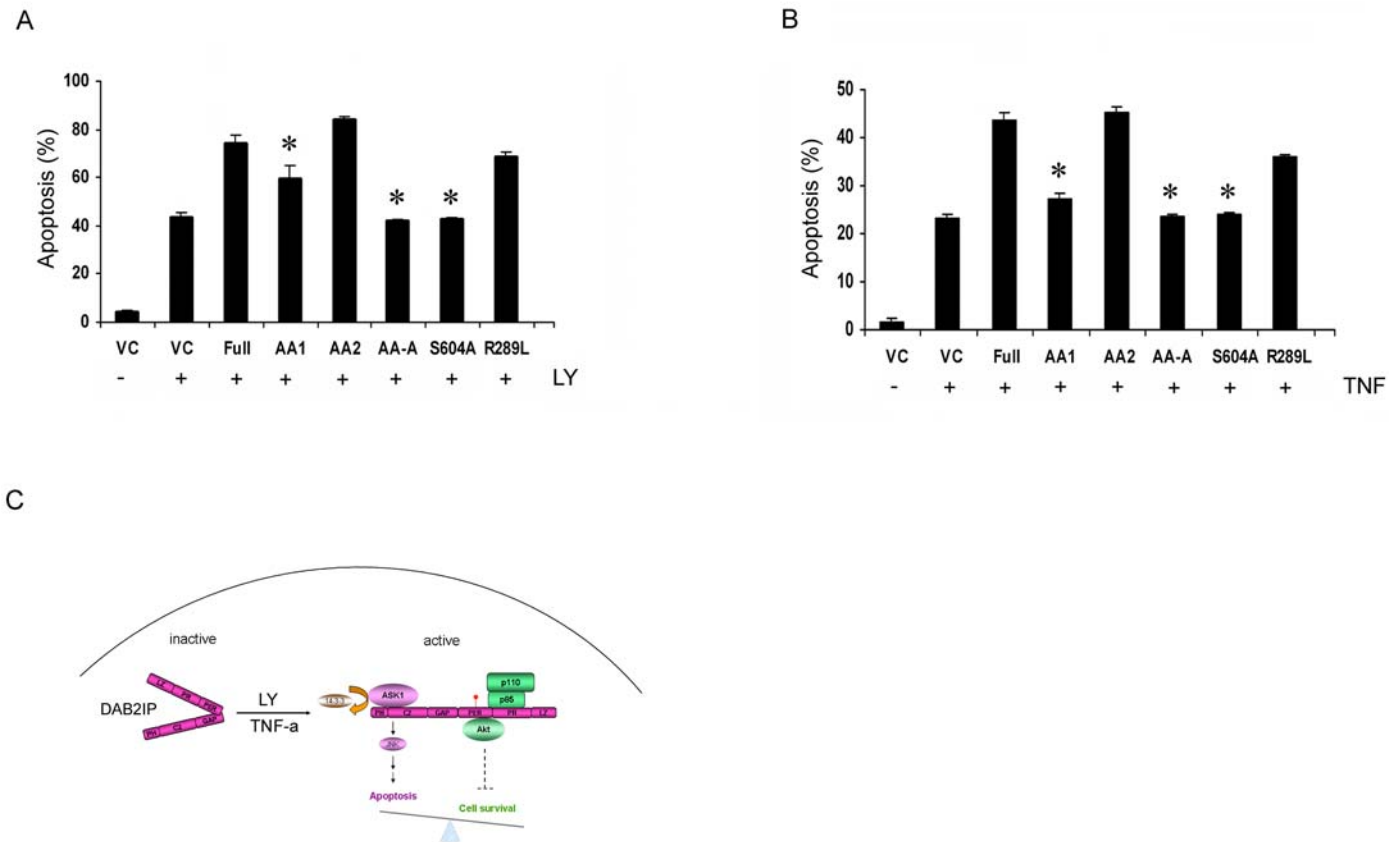# Fig 5. PR domain dictates DAB2IP-mediated PI3K/Akt inhibition and ASK1 activation
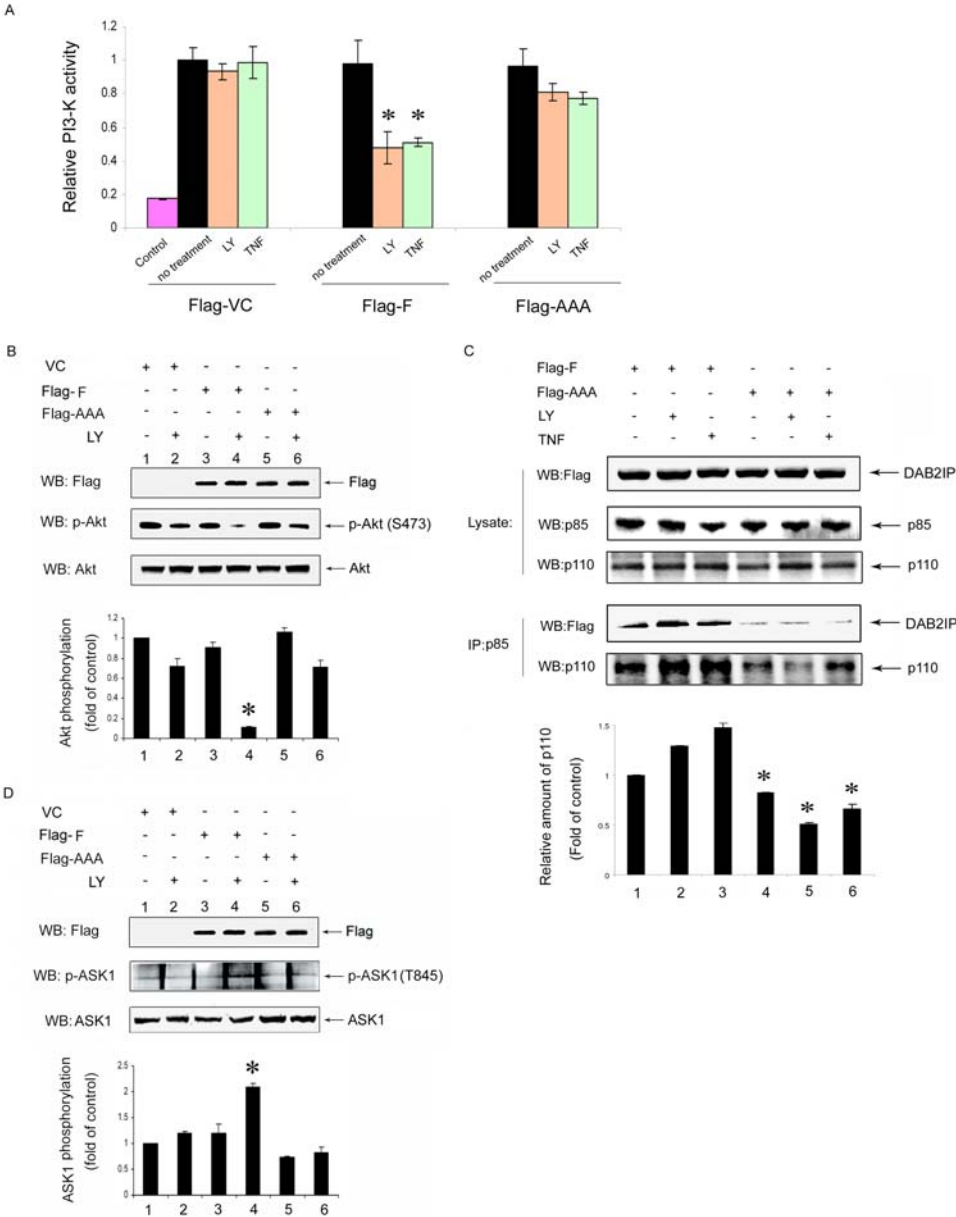
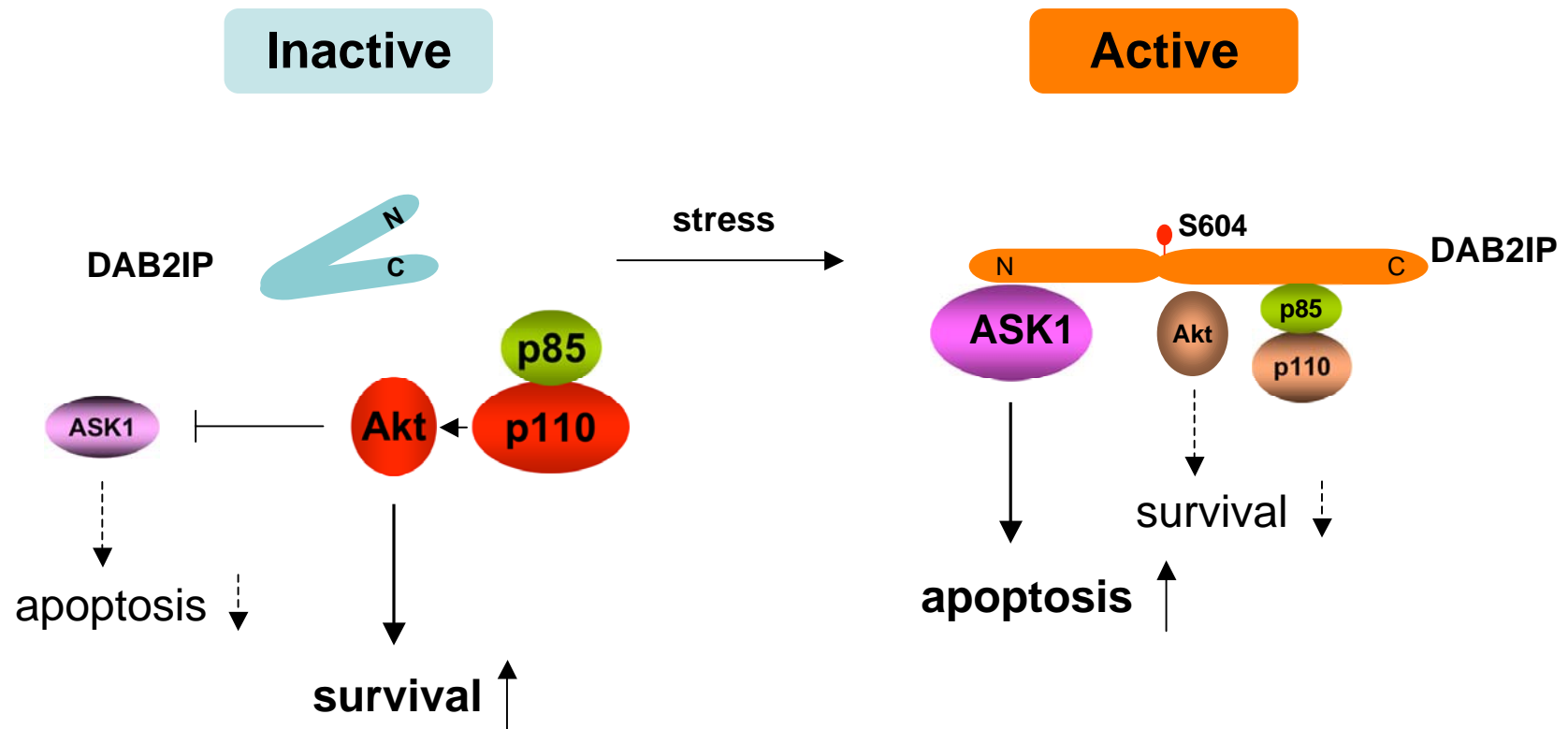Fig 6. ASK1-mediated S604 phosphorylation enhanced DAB2IP interaction with p85 and Akt
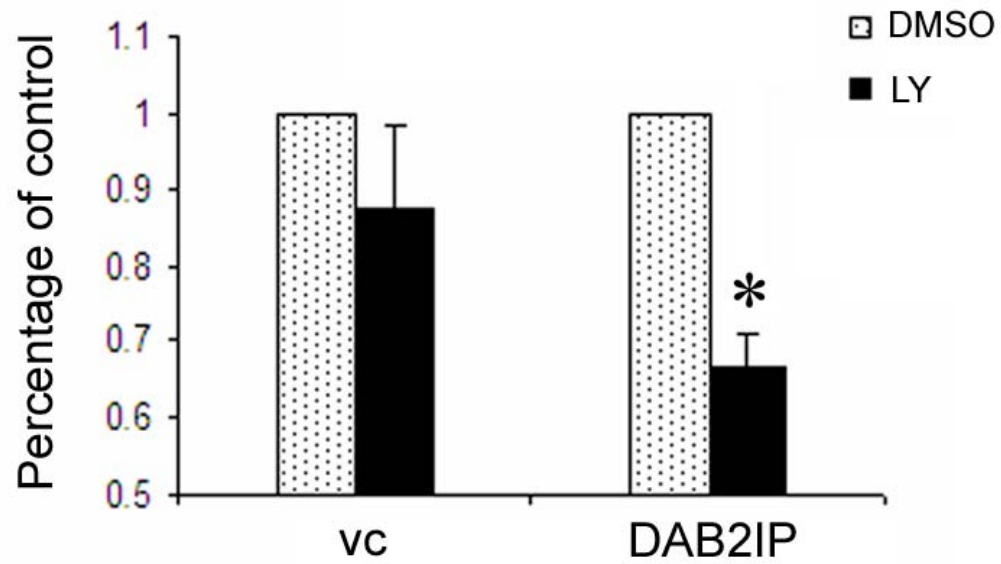
Fig 7. DAB2IP interacts with Akt through PER domain
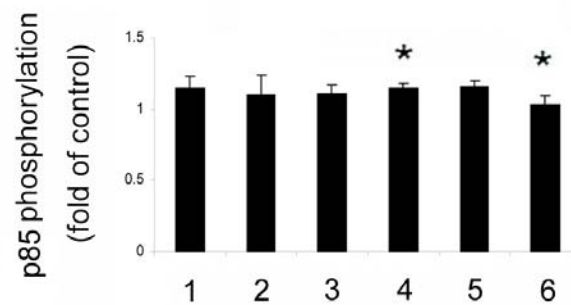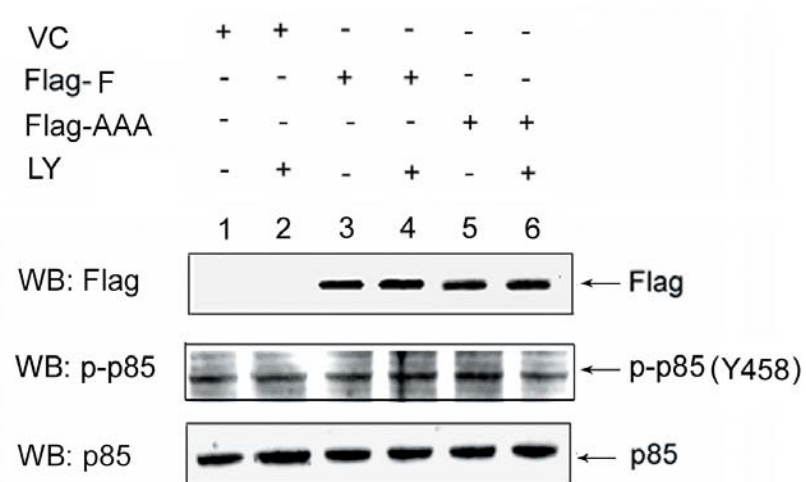
Fig 8. PR domain dictates DAB2IP-mediated apoptosis
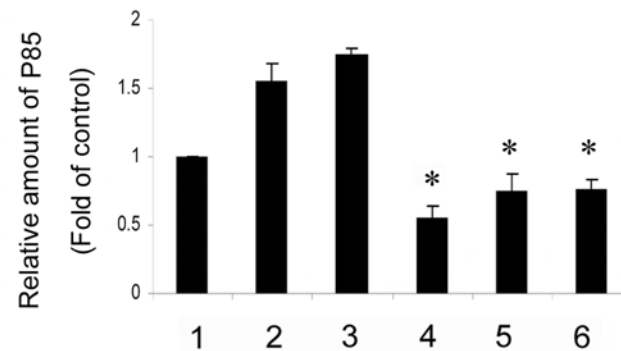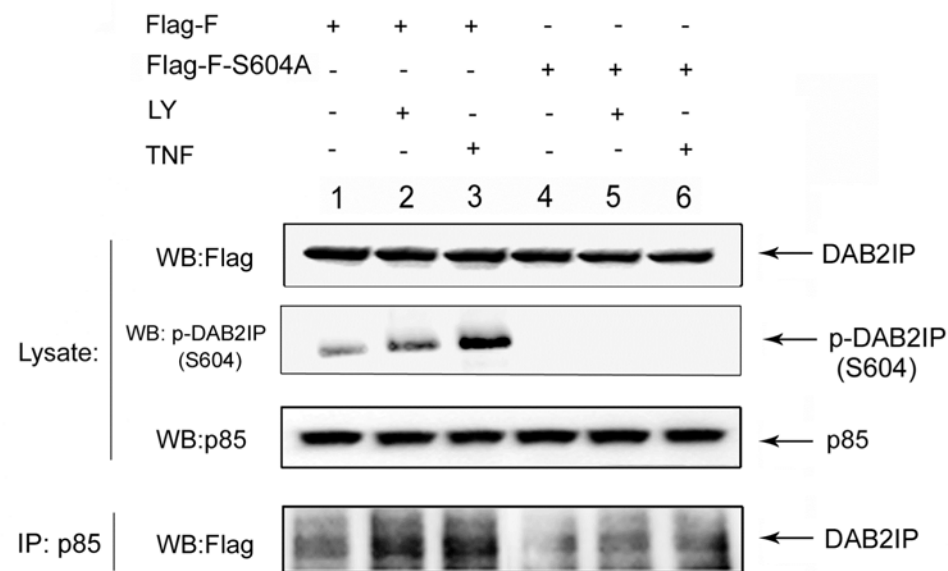
Fig S1. DAB2IP promotes cell death

Fig S2. DAB2IP has no effect on p85 phosphorylation

Fig S3. Searching results of binding sites in PER domain

Phosphoserine/threonine binding group (pST_bind)				
14-3-3 Mode 1			Gene Card YWHAZ	
Site	Score	Percentile	Sequence	SA
S604	0.3522	0.22%	PSPARSS**S**YSEANEP	2.006
Src homology 2 group (SH2)				
Fgr SH2			Gene Card FGR	
Site	Score	Percentile	Sequence	SA
Y605	0.4865	0.89%	SPARSSS**Y**SEANEPD	1.512
Src homology 3 group (SH3)				
Cbl-Associated protein C-SH3			Gene Card **N/A**	
Site	Score	Percentile	Sequence	SA
P599	0.7109	0.58%	SSGVQPS**P**ARSSSYS	1.947
Cbl-Associated protein C-SH3			Gene Card **N/A**	
Site	Score	Percentile	Sequence	SA
P696	0.7344	0.81%	SEGAPGR**P**QLLAPLS	0.809
Basophilic serine/threonine kinase group (Baso_ST_kin)				
PKC alpha/beta/gamma			Gene Card PRKCA	
Site	Score	Percentile	Sequence	SA
T675	0.4597	0.70%	VNLAGLA**T**VRRAGQT	0.785
Akt Kinase			Gene Card AKT1	
Site	Score	Percentile	Sequence	SA
S604	0.5561	0.56%	PSPARSSS**Y**SEANEP	2.006
Acidophilic serine/threonine kinase group (Acid_ST_kin)				
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S602	0.4827	0.21%	VQPSPAR**S**SSYSEAN	1.71
Proline-dependent serine/threonine kinase group (Pro_ST_kin)				
Cdk5 Kinase			Gene Card CDK5	
Site	Score	Percentile	Sequence	SA
S598	0.4929	0.83%	RSSGVQP**S**PARSSSY	2.516
Cdc2 Kinase			Gene Card CDC2	
Site	Score	Percentile	Sequence	SA
S598	0.4944	0.49%	RSSGVQP**S**PARSSSY	2.516
Phosphotyrosine binding group (PTB)				
Shc PTB			Gene Card SHC1	
Site	Score	Percentile	Sequence	SA
Y709	0.5485	0.43%	LSFQNPV**Y**QMAAGLP	0.714

Fig S4. S604 phosphorylation enhanced DAB2IP interaction with p85

Fig S5. S604 phosphorylation of endogenous DAB2IP